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Leon Tumerman and
Byron H. Webb

Coagulation of Milk and Protein Denaturation

THE COLLOIDAL STABILITY OF MILK

The coagulability of milk constitutes both an asset and a liability. Without it, there would be no cheese or cultured products as we know them. Coagulation, to produce a soft type of curd, with low curd tension, is considered a prerequisite to mammalian digestion of milk. Viscosity development and coagulation must always be controlled and sometimes prevented during dairy product manufacture. Incipient coagulation produces a desirable thickening in ice cream mix or evaporated milk, but it may impart to sweetened condensed milk an excessive viscosity, culminating in actual gelation. While complete coagulation yields the curd from which cheese is made, this kind of change in sterilized or frozen milk results in an unusable product. Feathering in coffee cream and insolubility in dried milk are further manifestations of undesirable coagulation.

The coagulation of milk involves the formation of large structural aggregates of casein, or curd, from the normal colloidal dispersion of discrete casein micelles. In forming a coagulum the suspended phase may assume a gel structure, a partially coagulated grainy condition, or it may separate entirely from the whey serum as a flocculant precipitate, depending upon the specific environmental conditions that prevail during coagulation.

A variety of agents including alcohol, acids, salts, heat, freezing, irradiation, and proteolytic enzymes can induce coagulation of milk. Extreme concentration can also effect coagulation by compacting the micelles into an extensively modified serum electrolyte environment, as exists in moist milk powder or frozen milk. The coagulation of the casein in milk bears some superficial resemblance to the coagulation of other biological fluids, such as blood or egg white. However, the milk system presents many unique sta-

LEON TUMERMAN, Research and Development Division, National Dairy Products Corporation, Glenview, Illinois.

BYRON H. WEBB, Research and Development Division, National Dairy Products Corporation, Glenview, Illinois. Present address Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C.

bility characteristics related to the specific properties of the casein micelles, the composition of the milk serum environment and their mutual interactions.

Colloidal stability, milk protein denaturation and the effects of processing conditions on the coagulation of milk are discussed in this chapter. Related subjects including rennet coagulation and cheese chemistry are presented in Chapter 12, and coagulation in the frozen state is discussed in Chapter 14. A further discussion of physico-chemical problems may be found in Chapter 10.

Composition and Dispersion of the Casein Micelle

Casein is a heterogeneous phosphoprotein containing three electrophoretically distinct components, designated α, β, and γ casein, in order of decreasing electromobility. These comprise 80, 15, and 5% of whole casein, respectively, based on classical electrophoretic analysis.[146,424] Reassessment of their relative distribution by dye binding and fractionation techniques[237,254] has led to revised values of 60% α-, 32% β-, and 9% γ-casein. The significance of casein heterogeneity lies in the fact that micellar stability is derived from the composite interactions of its discrete components. Each of these possesses distinctive compositional, structural, and physicochemical properties. A number of components, not evident in the usual course of electrophoretic analysis, have been identified in whole casein, and more specifically in the α-complex. The identification and characterization, by Waugh and von Hippel,[435] of a calcium soluble component in the α-casein complex, designated κ-casein, and of a calcium insoluble component, α_s, is of particular significance and will be considered in a subsequent section on casein polymer interactions. Current developments in the area of casein resolution by paper electrophoresis of urea solutions of casein,[223,475] and starch-gel zone electrophoresis[427] indicate a remarkable complexity, particularly of the α-casein. The results of these methods of resolution indicate that casein may contain upwards of 20 distinct components! Whether all the components resolved under such procedures are true natural constituents, or constitute in some measure artifacts of the fractionation of casein, has yet to be ascertained.

The casein components are colloidally distributed as polydisperse stable micellar aggregates in association with calcium and phosphate, and lesser amounts of magnesium and citrate. The total complex, of undefined structure, is generally referred to as the calcium caseinate-calcium phosphate complex. The caseinate micelles may be considered as swollen, microscopic polyelectrolyte gels, containing in excess of 66% water, of which approximately one-fourth is chemically bound. The exact mechanism for the retention of the major proportion of unbound water in the micelles is not clearly understood. It has been variously attributed to osmotic forces imposed by the Donnan equilibrium requirements[230–233,461] and to a type of colloidal "charge swelling," akin to the swelling of gelatin and collagen in acids and bases.[203] From the sedimentation properties of the caseinate particles, the micellar volume calculates to be 4.0–4.5 times the volume of the dry framework, corresponding to a voluminosity of 2.8–3.2 ml per gm. of casein[78,91] The approximately spherical micelles have been estimated to range in size from 400–2,800 Å by electron microscopy,[281] 200–1,400 Å by ultracentrifugation,[387] 800–1,100 Å by light dispersion,[69] and 50–1200 Å by ultramicroscopy.[458] The dominant frequency of particle size, determined by electron microscopy, lies in the 800–1200 Å range, equivalent to an apparent molecular weight of 81–266 million. The extent to which casein is aggregated in milk can best be appreciated by the fact that the molecular weight of the casein monomers is only 15,000–25,000, according to current estimates.[421,434] Variation in the distribution of the individual casein components, as a function of micelle size, has been suggested by a number of investigators.[25,143,381,394] The further possibility of preferred micelle size grouping is indicated by centrifugal sedimentation characteristics.[91,466] The casein micelle population is estimated to be in the order of 10^{12} particles per cubic centimeter of milk, with an average free path of 0.36μ between particles.[458] The micelles are in constant kinetic motion, and in consequence of their closely packed condition in milk, the entire dispersion may be immobilized by cohesion of a relatively small proportion of the particles. Unrestrained, the growth of the casein micelles would lead to spontaneous coagulation of milk. However, in normal fresh cow's milk the colloidal dispersion is remarkably stable to extremes of temperature and concentration. It can sustain severe boiling and freezing conditions, and substantially recovers its normal dispersion after concentration, freezing, or dessication. Under sterile conditions, the integrity of the colloidal dispersion is maintained over protracted storage periods.

The forces that so effectively limit micelle size are obscure, but Waugh and von Hippel[435] suggest that they may derive from complex polymer interactions of the casein components, a subject that will be discussed in a later section.

Although the casein micelle exhibits unusual stability attributes within the "normal milk" environment, it is vulnerable to compositional changes in the milk related to the physiology of lactation, or induced by bacterial growth, and applied process treatments. Micelle size dispersion is modulated by a generally reversible equilibrium with the calcium, phosphate, citrate, and magnesium ions in the milk serum. Addition of multivalent cations to milk causes progressive aggregation of the casein micelles, evidenced by an increased centrifugability and turbidity, and a lower tolerance to heat, culminating in spontaneous coagulation. The addition of phosphate, citrate, or other calcium complexing anions, enhances micellar dispersion, a change manifested by reduced turbidity and sedimentation, and a commensurately increased heat tolerance. Hydrogen ion concentration and temperature are integral components in the complex reversible equilibria that bear on casein micelle stability. Casein hydrolysis by proteolytic enzymes or heat, and the formation of various heat-induced complexes between casein, serum proteins, and lactose, constitute essentially irreversible changes that profoundly alter micellar stability.

Effect of Hydrogen Ion Concentration on Casein Micelle Stability

In contrast to the milk serum proteins and native proteins generally, casein is markedly insoluble at its isoelectric point of pH 4.6, a value ascertained by solubility and electrophoretic criteria. Although, electrophoretically, the isoelectric point is rather sharply defined, coagulation of milk is usually initiated by acidification to a pH of 5.3, and is complete at pH 4.6. For practical purposes, it is more appropriate to refer to a region of isoelectric precipitation of casein. With diminishing salt concentration, the isoelectric point of casein solutions approaches a limiting pH value of 4.51.[272] The solubility of isoelectric whole casein is of the order of 0.11 gm. per liter of water at 25°C.[47] The isoelectric points of the several casein components are not identical. The respective values for α-, β-, and γ-caseins are pH 4.7, 4.9, and 5.8–6.0.[145] Consequently, acidification of milk to pH 4.6 yields a coagulum of whole casein, inclusive of the various casein components that comprise the micelle. The isoelectric point of casein is slightly increased in heated milk,[80,81,398] presumably due to its association with the denatured serum proteins, and under these conditions the coagulum constitutes a coprecipitate of casein and denatured serum protein. Methods employed in the commercial manufacture of casein by isoelectric coagulation, the properties of casein and its industrial utilization have been reviewed extensively elsewhere.[374,386]

Under the influence of increasing hydrogen ion concentration, the calcium phosphate is progressively dissociated from the micellar complex. At the isoelectric pH, calcium phosphate is wholly dissociated from the casein. Changes in the natural state of dispersion of the casein micelles, as a function of hydrogen ion concentration, have been assessed by ultracentrifugal,[24,32,75,315,316,332,382,388,421] microscopic,[163,164] and turbidimetric methods.[33–35,280,429,430] Measurement of the changes in the light reflectance of milk in the visible spectrum indicate that the mean micellar size decreases rapidly above pH 6.6, but is relatively independent of pH in the range 5.8–6.6.[34] Comparable results are obtained by viscosity measurements on milk which evidence only slight variation in the pH range 5.3–6.7, with a faint minimum at the natural pH of 6.6. However, large viscosity increases on the alkaline or acid side of this relatively stable pH zone constitute evidence of extensive micellar dispersion, and aggregation respectively.[78,450] Turbidimetric measurements of the coagulation of milk under progressively increasing hydrogen ion concentration,[429,430] further concur with the measurements made by reflectance and viscosity methods.

The acidity of milk varies with the individual cow and the stage of lactation, but predominantly as a result of lactic acid developed by bacterial metabolism of the lactose. The normal pH range of pooled fresh cow's milk is 6.4–6.8, with an average titratable acidity range of 0.145–0.165%. At this acidity level, the casein in milk is not intrinsically heat coagulable, withstanding 100°C. for upwards of twelve hours. However, its susceptibility to heat coagulation increases sharply with increasing hydrogen ion concentration and, at a developed lactic acidity of 0.25%, coagulation may be spontaneously initiated at temperatures as low as 82°C. With further acidity development to levels of 0.35, 0.40, 0.50, and 0.57%, the spontaneous coagulation temperatures decrease to 65, 38, 23, and 18°C., respectively.[251] The pH of milk may be lowered to the normal isoelectric region without incurring coagulation of the casein, if the temperature is maintained near 2°C. during acidification.[338] Zittle *et al.*[482] measured the coagulation rates of casein in buffer solutions containing calcium chloride, at pH 5.4–5.6, as a function of temperature. Turbidity changes, assessed by a light extinction procedure, indicate that different buffers are without specific effect on the aggregation process.

The distinctive insolubility of casein in the isoelectric region, and the susceptibility of its solutions to precipitation by multivalent cations, are properties related to the fine structure of the protein molecules. Casein as an amphoteric electrolyte derives its charge principally from the dissociation of its amino and carboxyl groups, which occur as a complex function of pH, temperature, ionic strength, and solvent composition. The phosphorus bound to casein, presumably as a phosphoric ester of the amino acids serine or threonine, is ionogenic and also contributes materially to the total charge on casein. In the isoelectric state the oppositely charged groups in casein are balanced by equal dissociation, and the net free charge is eliminated. On the alkaline side of its isoelectric point, as in fresh milk, the negatively charged groups of casein predominate, evidenced by its mobility toward the anode under an applied electrical field. The converse situation prevails in casein solutions acidic to the isoelectric value.

Solubility as well as a number of other properties including solution viscosity, hydration, and swelling generally evidence minimum values in isoelectric protein solutions. The nearly total loss of solubility incurred by casein is evidenced by few native proteins in the isoelectric condition. The residual solubility that isoelectric proteins maintain is generally ascribed to retention of a sufficient degree of hydration to prevent molecular association and coagulation, in the absence of a net electrical charge. For casein, this residual hydration is inadequate to prevent coagulation. The affinity of a protein for water would be expected to parallel a high content of charged groups, and generally, proteins with higher dissociation constants do exhibit greater solubility.[47] Though the proportion and distribution of polar and nonpolar groups should largely determine the solubility of a protein, no reliable correlation of structure with solubility has been established. Considering the ratio of ionic to nonpolar groups as a measure of polarity, McMeekin[252] calculated that α-casein with 291 ionic groups and 965 nonpolar groups per 10^5 gm., is potentially about twice as polar as β-casein with 219 ionic groups and 1567 nonpolar groups, contained in an equal weight of casein. Relative polarity determinations by Hipp *et al.*[145] based on the ratio of protein solubility in 50% alcohol to the solubility in water, concur with those derived from structural analysis. However, marked insolubility at their respective isoelectric points is characteristic of the several casein components, even though their amino acid composition differs considerably.[105] Furthermore, α-casein aggregates much more readily than β-casein although it bears a higher net charge.[421] The higher net charge of α-casein, evident from compositional analysis, is borne out by its higher electromobility values on both alkaline and acid sides of its isoelectric point. These results suggest that charge interactions may therefore be of less consequence to casein than is generally proposed for the coagulation of other proteins.[421,432] Waugh[432] proposed that the insolubility of casein may be more readily rationalized on the basis of the formation of thermally stable bonds, through interaction of a relatively few large nonpolar side chains, than through the more widely postulated charge interactions. In support of this proposal, calculations for the ratios of large nonpolar side chains to the total number of side chains for several proteins, including casein, are advanced as a basis for comparing their relative potential for such nonpolar side chain interactions. Accordingly, α-casein evidences the highest ratio, of 0.47, based on frequency of occurrence of the nonpolar side chains contributed by such amino acids as valine, leucine, isoleucine, and proline. For β-casein, β-lactoglobulin, serum albumin, and tropomyosin the ratios decrease to 0.37, 0.32, 0.29, and 0.24, respectively.[432]

The solubility of casein, in the absence of multivalent cations with which it forms micelles, increases sharply at pH values above the isoelectric point. This occurs as a consequence of the increasingly negative net charge acquired by progressive anionic dissociation, and the commensurate increase in hydration. Casein can be extensively solubilized as an alkali caseinate, forming stable solutions that are highly ionized and of considerable viscosity. The alkaline earth caseinates, such as calcium caseinate, are by contrast coarse colloidal dispersions of lower degree of ionization and hydration, coagulable by heat and rennin. The respective base binding capacity of casein for sodium hydroxide and calcium hydroxide is given by Sandelin[354] as 6.7×10^{-4} and 7.5×10^{-4} equivalents per gram at pH 7.0. Though alkali solubilized casein is highly hydrated and dispersed, the persistence of molecular aggregates sensitive to temperature and pH is revealed by ultracentrifugal sedimentation analysis.[68] Von Hippel and Waugh[421] observed that isoelectric precipitation effects changes in casein that lead to more stable aggregates in its solutions, than is evident in casein directly solubilized without exposure to the isoelectric condition. Such soluble casein, prepared from skimmilk by total removal of calcium at pH 6.6, 5°C., exists as polymers whose complete conversion to monomers is attained at pH 12.0. These monomers are characterized by an average sedimentation value $S_{20}°$ of only 1.18×10^{-13} sec. Calculations derived from their diffusion coefficient value, $D_{20}° = 7.11 \times 10^{-7}$ cm²/sec., a partial specific volume, $\bar{v} = 0.731$ and the $S_{20}°$ value cited, indicate an average monomer molecular weight of 15,000. The monomer-polymer equilibrium is pH and temperature dependent, and completely reversible. Readjustment of the pH from 12.0 to 7.0 restores the ultracentrifuge pattern characteristic of the polymer aggregates of the soluble casein. At temperatures near 0°C., β-casein persists as a monomer of 1.3 S while the α-casein polymers attain a sedimentation constant of approximately 5.8 S. Interaction of α- and β-caseins to form a polymer complex occurs with increasing temperature at pH 7.0. Evidence of such an interaction is deduced from the disappearance of the β-peak, and formation of polymers whose sedimentation constants increase from 4.4 S at 4°C., to 9.3 S at 32°C.[421,435]

Effect of Electrolytes and Temperature on the Stability of the Caseinate Dispersion

The salt components of milk, of primary significance to the stability of the caseinate dispersion, are calcium, magnesium, citrate, and phosphate. These are distributed in both soluble and colloidal form, and maintained in a highly labile equilibrium with the caseinate complex. The addition or withdrawal of any of these salt components initiates a redistribution that alters stability. Equilibrium is further influenced by the hydrogen ion concentration and by temperature. Of the total calcium in milk, averaging 125 mgm% (0.03*M*), approximately two-thirds is colloidally dispersed and one-third is held in true solution. Ionic calcium, at a level of 0.002*M*, comprises less than ten per cent of the total calcium in milk while the bulk of the calcium is retained in nondissociated complexes with the phosphate, citrate, and protein components. Under the influence of increasing hydrogen ion concentration, the calcium phosphate is progressively dissociated and at a pH value of 5.2, substantially all the calcium and phosphate is soluble. Increasing temperature, on the other hand, drives calcium phosphate into the colloidal state, owing to the inverse temperature-solubility property of the salt and its near saturated condition in milk. The size of the caseinate aggregates in milk is increased by the addition of calcium and reduced by ion exchange removal of calcium or by the introduction of a number of calcium sequestrants such as phosphate, citrate, oxalate, fluoride and ethylenediamine tetraacetate.[23,35,67,421,433–435]

Changes in the size dispersion of the caseinate micelles as a function of salt balance, pH, and temperature variables are evidenced by altered centrifugal sedimentation rates, light reflectance properties, and viscosity changes. Within normal ranges of pH, salt, and protein content, the micellar size dispersion is nominally affected by temperature. From changes in the light reflectance of milk, Burton[33,35] concluded that increasing temperatures up to 50°C. effect a gradual increase in the mean size of the caseinate particles, which is reversible on subsequent cooling. Nichols *et al.*[280] deduced from optical analyses that changes in the caseinate dispersion of milk, heated at temperatures up to 95°C., are negligible. Decreasing nitrogen sedimentation rates in milks heated in the range of 88°–100°C., imply a moderate disaggregation of the micelles.[75,332] However, the extent of such change in the micellar dispersion is obscured by simultaneous changes in viscosity, micellar hydration, and density, and the interactions incurred between casein and the denatured serum proteins. Under conditions of high temperature–short time heat treatment (HTST), some degree of micellar aggregation is apparent.[466] HTST heat treatments, comparable to those employed in milk sterilization (82°C.-30 min. preheat, 150°C.-2 sec. sterilization heat), cause an increase in the ultracentrifugal sedimentation constant to a value of $2{,}277 \times 10^{-13}$, from 440×10^{-13} for the raw milk control. These sedimentation values, measured a few hours after heat treatment, decrease, on storage at 37°C. for 71 days, to 948×10^{-13}, indicative of partial disaggregation. Hostettler and Imhof,[163] by direct observation with the electron microscope, concluded that the casein micelles in milk, heated to boiling for short periods, remain essentially unaltered in size and shape. However, disaggregation becomes evident when the heating period at 100°C. is extended to 15 min. The coagulation of fluid milk by heat would not, therefore, appear to relate to any direct influence of increasing temperature, but rather to secondary changes in the milk system incurred by sustained exposure to high temperatures.

Waugh and associates[421,434] have demonstrated that changes in the caseinate dispersion in milk, due to alterations in calcium, phosphate, and pH, derive from a reversible equilibrium between centrifugable micellar casein, and a soluble casein portion of less than ten per cent of the total. The addition of $0.07M$ calcium chloride to skimmilk at 5°C. converts substantially all the soluble casein into centrifugable micelles.[25,421] The soluble casein in milk occurs as monomer and small polymer units of the casein components. A fivefold dilution of skimmilk with water, or saline solution, effects a threefold increase in the noncentrifugable casein, a result compatible with the qualitative observations that dilution of milk causes micellar disaggregation, as viewed in the electron microscope.[162] Micellar disaggregation normally incurred by dilution can be averted by restoring the calcium concentration. The equilibrium level of soluble, noncentrifugable casein is also temperature dependent. A residual level of 6% soluble casein is found in skimmilk centrifuged at 20°C. for 180 min. (50,000 g), while at 4°C. the soluble casein level increases to 15%.[25]

The progressive addition of calcium to milk greatly increases micellar size and sedimentation rate of the casein, and reduces the stability of the milk to heat. This effect is amplified by increasing temperature and pH, and suppressed by calcium complexing anions or increasing alkalinity. Since the addition of salts such as calcium chloride simultaneously lowers the pH of the milk, the coagulating effect must be considered a composite result of the increase in both calcium and hydrogen ion concentration. D'yachenko[67,68] reports that a maximum casein particle size is attained at 25°C., at a pCa value of 1.5, representing the negative logarithm of the calcium ion concentration. At this pCa, coagulation is incurred at a temperature of 40°C., while at a pCa value of 2.0, the coagulation temperature is raised to 80°C.

Milks of abnormally high natural calcium ion concentration, a defect related to irrational feeding practices, malnutrition of the cow or defective soil and pasture conditions, have been studied with respect to calcium variation and heat stability. This type of abnormality, originally observed in occasional samples of Netherlands milk, is manifested by an unusual susceptibility to coagulate on boiling and has been termed the Utrecht defect. Such milks, normal in respect to bacterial count, acidity, and general composition, possess a higher concentration of ionic calcium.[361]

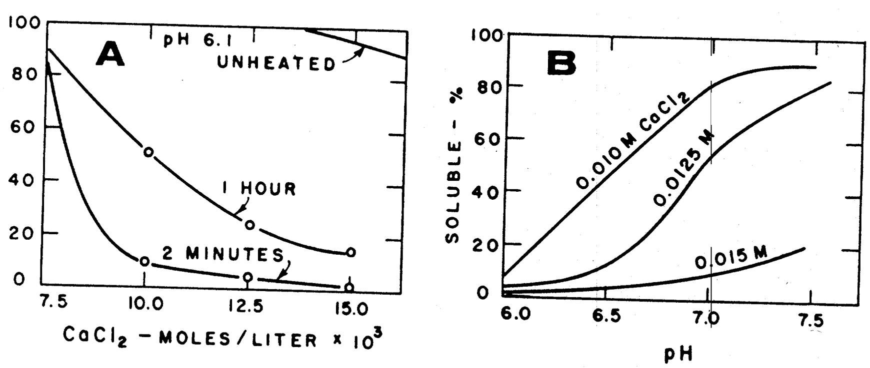
Coagulation of casein is generally incurred by the addition of significantly lower concentrations of cations of increasing charge. Its coagulation by monovalent salts, such as ammonium sulfate or sodium chloride, requires half and full saturation levels, respectively. Coagulation of this type, classified as a salting-out effect, relates to a reduction in the solubilizing interactions between the water molecules and polar groups of the proteins. Calcium and other polyvalent ions, however, interact strongly with casein, generally with a considerable loss of solubility. At pH values below the isoelectric point, where casein as an ampholyte bears a predominantly positive charge, calcium binding is negligible. Conversely, on the alkaline side of the isoelectric point, calcium binding by the negatively charged casein increases progressively in apparent relation to the increasingly available binding sites.

Attempts to correlate casein coagulability with the extent or reversibility of calcium binding have not yielded definitive results. Conclusions relative to the increased calcium binding capacity of rennetted casein (paracasein), drawn by some investigators, do not appear to be justified on the basis of more recent work.[56,417,418] Calcium binding measurements by equilibrium dialysis[337] suggest a possible correlation between irreversible binding of calcium and coagulation of casein. Calcium binding, by α, β, and whole caseins at pH 7.2, exhibits complete reversibility until visible coagulation is incurred, while kappa free α-casein appears to bind calcium irreversibly at this pH. Increasing ionic strength attenuates calcium binding, suggestive of electrovalent or ionic type bonding.[337]

The binding capacity of whole casein for calcium, measured at pH 7.0, is 7.5×10^{-4} equivalents per gram casein, according to Sandelin.[354] Tessier and Rose[396] find a maximum of 38.5M calcium bound per 10^5 gm. casein at pH 6.95, by analysis of the ultrafiltrate separated from colloidal casein-calcium hydroxide suspensions. Relatively minor changes in binding capacity are observed over the temperature range of 25°–93°C. These investigators report a decline in the dissociation constant of calcium caseinate, upon aging of the dried casein used in the analytical determination for calcium binding. The dissociation constant pK = 4.52, of freshly prepared freeze dried casein at pH 6.95, declines at least 0.6 units during the first month and then continues to recede more slowly to a final pK value of 3.0 over several months of storage. This observation is of interest with respect to a prior disclosure by Nitschmann *et al.*[285] that dry casein, during protracted storage, becomes progressively sensitized to calcium, eventually acquiring the properties of a rennetted casein. Tessier *et al.* further note a higher calcium affinity of freeze dried casein over commercial or solvent dried caseins. pK values for aged, freeze-dried casein generally concur with those reported by other investigators. pK values of 2.66, 2.39, and 3.34 have been measured by membrane electrode,[37] ultracentrifugal,[40] and equilibrium dialysis[481] methods respectively.

The coagulation of calcium caseinate dispersions by heat bears a general qualitative similarity to that of the natural micellar dispersion in milk, with respect to the influence of pH, salt concentration, and temperature. However, colloidal dispersions of calcium caseinate generally exhibit lower heat stability than the micellar casein of milk. The coagulating effect of heat on calcium caseinate, under conditions excluding structural changes in the casein, may partially reflect its decreasing solubility with diminishing ionization. The heat stability of colloidal dispersions of casein in calcium hydroxide solutions exhibits a dependency on the ratio of soluble calcium to soluble phosphate, as well as the degree to which the casein is saturated with calcium.[396] In the pH range 6.5–7.5, at calcium levels above 7.5 mM/liter, the heat stability of colloidal calcium caseinate bears an inverse relationship with the degree to which the casein is saturated with calcium. With increasing bound calcium values, representing 71, 82, 97, and 99% saturation of the casein at pH 7.5, heat stability values decrease from 26 to 16.5, 1 and 0.5 min. respectively. The heat stability of complex casein suspensions, containing added phosphate, bears an inverse relationship to the ratio of ionic calcium/soluble phosphate.

Zittle *et al.*[478] found that viscosity of two per cent calcium caseinate dispersions, heated to 90°C. for one hour, increases at calcium concentrations exceeding 0.012M, due to formation of colloidal casein precipitates. A viscosity decrease on aging, indicative of partial resolution, bears analogy to the inverse solubility-temperature relationship of calcium phosphate. At calcium concentrations lower than 0.012M, heated calcium caseinate solutions develop an opalescence that is substantially reversible on cooling. Typical solubility relationships of heated calcium caseinate systems are reproduced in Fig. 58.



From data by Zittle et al.[478]

FIG. 58. PRECIPITATION OF 2% SODIUM CASEINATE SOLUTIONS HEATED 1 HR. AT 90°C. AT VARIOUS pH LEVELS AND CALCIUM CHLORIDE CONCENTRATIONS

Solubility measured at 25°C., immediately after cooling, unless otherwise indicated.

In an extension of these studies, Zittle and associates[481,482] have assessed the influence of a number of variables, including pH, temperature, and salt concentration on the rate of aggregation of calcium caseinate dispersions. The aggregation rate curves for calcium caseinate at lower temperatures, 25°–35°C., are comparable to those reported by D'yachenko[67,68] but the maximum aggregation stated to occur at $0.01M$ calcium concentration could not be confirmed. At a fixed pCa the rate of aggregation of casein increases rapidly with decreasing pH values, a relationship implying decreased hydration of the protein aggregate. Rate changes in the aggregation of calcium caseinate as a function of temperature are characterized by high temperature coefficients of similar magnitude to those reported by Berridge[20–22] for the rennin clotting process. Temperature coefficients for ten degree intervals, calculated from the Arrhenius equation, are 19 for whole casein and 14 for α-casein. The rate of aggregation is further influenced by the ratio of calcium to casein. At a calcium concentration sufficient for maximum charge neutralization of the casein molecule by calcium binding, the rate becomes a function of the protein concentration.

Much of the research on the stability of milk, as evidenced by the preceding survey, constitutes an empirical assessment of the influence of many interrelated variables on the dispersion of both the natural caseinate complex in milk, and simulated calcium caseinate colloids. Recognition of the heterogeneity of whole casein, and particularly of the α-casein fraction, has added a new dimension to our understanding of casein micelle stability and of the coagulation process. Waugh and associates have disclosed, in a series of remarkably incisive experimental studies on the components of casein, the dominant influence of their polymer interactions on micellar stability.[421,422,433–435]

Casein Polymer Interactions and Micelle Stability

Numerous concepts have been advanced to accommodate observations relating to both the natural stability of the casein micelles in milk, and their marked susceptibility to clotting by traces of renneting enzymes. One proposal held that the more hydrophillic serum proteins may function as protective colloids for the casein,[3,414] but this has proved inconsistent with the stability of serum protein-free milk,[330] and with the demonstrated specificity of rennin toward casein. The electro-kinetic potential and water binding properties of caseinate dispersions, although intensively studied, have afforded meager insight into the stability characteristics of milk. The destabilization of calcium caseinate sols by calcium chloride has been attributed to a decrease in zeta potential, while coagulation by rennin has been viewed primarily as a dehydration process, with a subordinate decrease in potential.[119,120] Based on measurements of the zeta potential of caseinate sols as a function of protein and calcium concentration, a critical value approaching -10 mv. was proposed as a minimum potential required for the stability of a 2.5% dispersion at 30°C. and pH 6.6. However, changes in water binding by casein, as a function of varied processing conditions, show little consistent correlation with micellar stability. The susceptibility of milks to coagulation by alcohol also evidences poor correlation with changes in bound water.[322] Furthermore, process treatments that stabilize milk to coagulation effect no consistently related change in the bound water content.

The aggregation of calcium caseinate with increasing calcium concentration has been attributed to cross linkages established by the divalent calcium,[315,316] presumably by initial binding at the phosphoric and free carboxyl groups of the casein. Attempts to correlate rennin clotting with changes in calcium binding by the paracasein have, however, led to conflicting results.[56,417,418] Eilers[77] considers that the calcium caseinate in milk is comparable to that of an incipient coacervate in which the colloidal caseinate assumes a less highly charged condition by the formation of a double salt with tricalcium phosphate, but still sufficiently solvated and stable toward further flocculation. Similarly from the dispersion characteristics of calcium caseinate sols in urea solution, it has been concluded that the aggregation of casein micelles evolves from a diminished charge and hydration of calcium caseinate.[203] Such aggregation is believed to be induced by an ionic charge effect rather than by calcium cross linkage. Waugh and von Hippel[435] stress the fact that "the casein micelle population in milk is stable although the micelles are highly polydisperse, extremely variable in the number of polymers per micelle, with the smallest of micelles estimated to contain thousands of casein monomers." Waugh[434] therefore rejects the proposal that stability of the caseinate dispersion evolves from hydration and electrostatic interactions, a view that would be acceptable only if the caseinate micelles were sufficiently insoluble to deprive the larger micelles from growing at the expense of the smaller ones. This is held untenable since the micellar casein components have been demonstrated to be in equilibrium with the noncentrifugable, soluble casein components in milk.[421,434] Alternatively, he has proposed that casein micelle stability must derive from specific polymerization interactions of the individual components of casein, moderated by the ionic environment and temperature.

Early evidence of casein heterogeneity,[226,291] based on solvent fractionation, directed attention to the possibility that one of the components of whole casein might exercise a protective colloid effect on the complex. With great insight, Linderstrom-Lang,[224] as early as 1929, conceived that the clotting of milk by rennin could involve the modification of some specific protective component within the casein complex, thereby exposing a calcium sensitive residue to the coagulating action of the serum calcium. With the advent of electrophoretic analysis, three components, designated α-, β-, and γ-caseins, of distinctive electromobility values, were identified and suitable fractionation methods were developed for their recovery and characterization.[145–147,256–258,382,431] That the β- and γ-components in whole sodium paracaseinate remain intact, while the α-component evidences a partial split electrophoretically, was demonstrated by Nitschmann,[283] and Cherbuliez and co-workers.[41] Emerging evidence of the further possible heterogeneity of α-casein has served to focus attention on this complex as the potential source of a protective component. However, assignment of the protective colloid role, postulated by Linderstrom-Lang, to the α-casein, proved uncertain due to conflicting evidence relative to the specificity,[145,252] and hydrolytic function[285] of rennin. Pyne,[328] referring to the fact that α-casein is the major component of whole casein, stressed the inadequacy of its characterization as a protective colloid. The percentage of the presumed protective fraction was estimated to be less than 20% of the whole casein.[284]

Waugh *et al.*, in a succession of publications initiated in 1955,[421,433–435] disclosed the isolation and characterization of a component from the α-casein fraction, designated κ-casein, which bears very substantial evidence of functioning as a key micelle stabilizing factor, and as the primary site of rennin action. κ-casein constitutes, by preliminary estimates, approximately one-fifth of the total α-casein complex.[435] Accordingly, the relative concentrations of the casein components are tentatively re-estimated as 55% α, 30% β, and 15% κ-casein. α-casein, free of the κ-component, has been designated α_s-casein. κ-casein occurs as polymer aggregates in solution at pH 7.0, with a sedimentation constant of approximately 13.5 S, dissociating reversibly at pH 12 to monomers of 1.3 S.

Molecular weight values currently assigned to the monomer forms of α_s, β-, and κ-casein components are 23,300,[434] 24,100,[382] and 16,300,[434] respectively. The phosphorus content of purified κ-casein isolated by Waugh (0.19%), is very appreciably less than that of either α_s-casein (1.1% P), or β-casein (0.61% P), but its electrophoretic mobility is comparable to that of α_s-casein.

Isolation of an appreciably enriched κ-casein fraction from its natural complex with α_s-casein was accomplished by subjecting soluble casein preparations to the action of 0.25–0.4M calcium chloride at 37°C.[433–435] At these relatively high calcium concentrations, approximately 8 to 13 times the level in skimmilk, the α_s-κ complex tends to dissociate. The α_s-casein, deprived of the protective influence of κ-casein, precipitates together with β-casein, due to their insolubility, while κ-casein which is extraordinarily insensitive to calcium is retained in the supernatant. Briefly, the fractionation entails a centrifugal recovery of the casein micelles from skimmilk at pH 7, re-solution with added citrate, followed by dialysis. The resulting calcium-free soluble protein has been termed first cycle casein and its solutions, upon addition of 0.25–0.40M calcium, yield an insoluble precipitate termed second cycle casein. This precipitate consists of calcium α_s-caseinate and calcium β-caseinate while the supernatant "fraction-S" is predominantly κ-casein. Through further refinements, κ-casein preparations of approximately 90% purity, established by ultracentrifugal and electrophoretic criteria, have been prepared.

Considerable insight into the composition and stability of the casein micelle is afforded by Waugh's investigations into the polymerization interactions of these fractionated α-casein components. The essential disclosure resulting from such studies lies in the fact that an apparently stoichiometric interaction between α_s- and κ-casein, in a preferred ratio of 4:1, leads to the formation of stable micelles in the presence of calcium at 37°C. This favored ratio is equivalent to the estimated natural distribution of the α_s and κ-caseins in milk. In the absence of calcium, polymer complexes between α_s and κ-caseins are formed, rather than micelles, but the same ratio prevails in either case. The polymer interaction in the absence of calcium is characterized ultracentrifugally by the disappearance of the individual sedimentation peaks of α_s- and κ-caseins and their replacement by a single α_s-κ casein polymer complex with a sedimentation coefficient of 7.5S. At low temperatures, e.g., 2°C., the complex formed from mixtures of α_s- and κ-casein appears to be unstable and dissociates on addition of calcium to yield a precipitate of calcium α_s-caseinate. However, the α_s-κ

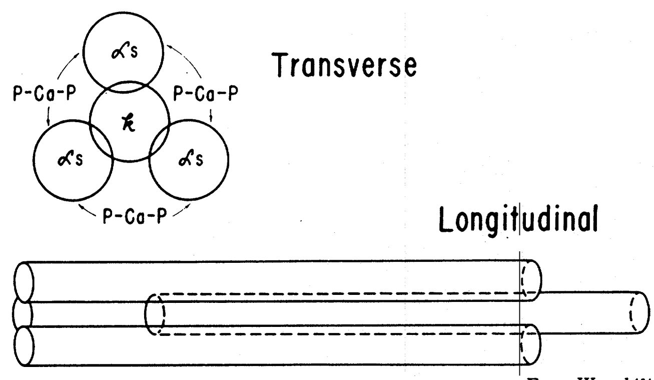
complex at 37°C., forms micelles upon addition of calcium, that retain their stability on subsequent cooling to 2°C., or on further addition of calcium. Waugh infers from the temperature dependence of the system that "only at the higher temperature does the structure of the α_s-κ casein complex permit the incorporation of calcium into stable intra-complex ion bridges, presumably by saturation of those calcium binding sites that would otherwise engage in inter-complex association."[435]

The formation of stable micelles requires the presence of an adequate amount of κ-casein and further depends on the sequence of introduction of the casein components and calcium, pH, temperature, and ionic strength of the system. Mixtures containing an excess of α_s-casein over the preferred ratio to κ-casein, precipitate in the presence of calcium. Without κ-casein, combinations of the several other components merely form precipitates, in accord with their solubility values in calcium solutions. The solubility of α_s-casein in gm. per liter of $0.03M$ calcium is, according to Waugh,[433] 0.03 and 0.17, at 4°C. and 37°C., respectively. β-casein, whose solubility is highly temperature dependent, is soluble at 4°C., but at 37°C. its solubility is reduced to only 0.2. In contrast, κ-casein is fully soluble over the reported temperature range, forming polymers of uniform size with a sedimentation coefficient of 13.5 S, uniquely stable to calcium and ionic strength as well as temperature.

A minimum calcium concentration of $0.03M$ is required for the spontaneous formation of stable micelles in solutions containing α_s- and κ-casein mixtures (weight ratio of 4), dialyzed skimmilk, or first cycle casein, at pH 6.5–7.0 and 37°C. Precipitates consisting largely of α_s-casein form as the calcium concentration is increased beyond $0.05M$, and attain a maximum at $0.25M$ calcium. Micelle formation, as evidenced by the development of opalescence upon addition of the cation to first cycle soluble casein solutions, is initiated at a threshold concentration specific to the divalent cation. The median divalent cation concentrations effective for micelle formation at 37°C., pH 6.5–7.0, are for calcium, copper, strontium, and barium, $0.03M$, zinc $0.007M$, and manganese $0.015M$.[433] Magnesium appears uniquely incapable of micelle formation, does not precipitate α_s- or κ-caseins, and is antagonistic to micelle formation by calcium. Cu, Ni, Co, Mn, Cd, and Zn confer notably greater stability to their α_s-κ complexes. The casein complex diluted in the presence of magnesium does not, therefore, exhibit the extensive dissociation evident in comparably diluted systems containing calcium.

While the casein components are individually susceptible to conversion by rennin to their respective para caseins, the unique changes in the properties of the rennetted kappa component implicate it as the primary site of enzymatic clotting.[434] Its rate of conversion by rennin to para-k-casein is very significantly greater than those of α_s- and β-caseins and is comparable to the clotting time for skimmilk. Of primary significance, the calcium sensitivity pattern of the several casein components is sharply reversed upon conversion by rennin to their paracasein form, and para-κ-casein acquires a singularly high insolubility in the presence of calcium. Furthermore, para-κ-casein is devoid of micelle stabilizing capacity, but addition of intact κ-casein to previously renneted casein restores its ability to form stable micelles with calcium.

Waugh[434] visualizes a tentative structure for the α_s-κ casein complex, based on available evidence, that comprises three α_s-casein molecules positioned peripherally around a single kappa molecule, in longitudinal alignment as in a cluster of four rods (see Fig. 59). Projection of an end segment of the central κ-casein



From Waugh[434]

FIG. 59. PROPOSED STRUCTURE OF α_s—κ-CASEIN

molecule, which is considerably shorter than the surrounding α_s-molecules, is further visualized as providing the requisite access by rennin. The primary polymer formation is assumed to derive from formation of hydrogen bonds, and interactions involving the abundant nonpolar side chains on the casein molecules. The phosphate groups on the three peripheral α_s molecules, conceived to be brought into alignment by the primary interaction of the κ- and α_s-caseins, could form a highly stabilized structure by cross linkages formed through calcium.

A number of investigators have advanced evidence in confirmation of the presence of a calcium insensitive, κ-casein component in the α complex, and have further expanded upon its composition, properties, and interaction with renneting enzymes.[25,235,308] The isolation and partial characterization of additional minor calcium insensitive casein components from the α complex, attests to the remarkable heterogeneity of casein.[235,253] A λ-casein component, isolated by Long *et al.*,[235] was estimated to constitute only 15–20% of a crude κ-casein preparation. While λ-casein appears to bear some superficial resemblance to κ-casein, it is reported to contain 1.18% phosphorus and, in contrast to κ-casein, shows no visible reaction with rennin. While further research on the resolution of casein may enlarge upon the number of identifiable components, and may indeed divulge additional polymer interactions as anticipated by Waugh,[433] the role of κ-casein as the essential micelle stabilizing element adequately accommodates currently available evidence.

The emerging breakthrough in the colloid chemistry of the caseinate micelle represents a resource, for future research and dairy product technology, of inestimable value. Its immediate impact on the rennin clotting mechanism, a process so vital to cheese technology, is already evident in the extensive research underway to probe the structure of κ-casein and the macropeptides released from it during clotting. Furthermore, the disclosure of a stabilizing component critical to casein micelle stability must inevitably lead to a reassessment of the various technological problems in which milk product coagulation is either an asset or a detriment.

A few of the more important problems, in which the chemistry of κ-casein as a micelle stabilizing component could conceivably be at issue, bear mention at this point. Is the micelle stabilizing capacity of κ-casein altered by process treatments involving heat, concentration, prolonged storage, freezing and dessication, and are such changes related to the heat and storage stability of the milk products? Do the benefits derived from forewarming of milk, with respect to improved heat tolerance of its concentrates, relate in any way to heat induced stabilizing interactions between κ- and α_s-casein, or with the denatured serum proteins? To what extent do natural variations in the content and distribution of κ-casein in milk relate to the variable stability of milk products during processing and storage? And finally, how does the electrolyte balance influence the polymer interactions of κ-casein, under process conditions that alter the stability of milk?

MILK PROTEIN DENATURATION

The preceding section has dealt largely with the equilibrium reactions that contribute to the stability characteristics of the caseinate dispersion in milk. However, the imposition of numerous irreversible changes in the milk system, by a variety of applied process treatments, is frequently a decisive factor in the stability of the milk colloids. Heat sterilization of concentrated milk, for example, may simultaneously evoke a partial dephosphorylation of casein, denaturation of the serum proteins, assorted interactions among the lactose, casein and serum protein constituents, an increase in acidity derived from multiple sources, and partially irreversible changes in the salt equilibria, all of which bear on the coagulation process to varying degrees. Such induced changes, further superimposed on the natural variability in milk composition, contribute to the unpredictability of its colloidal stability. These factors frequently obscure the underlying cause of coagulation, and anomalous results are not uncommon. Accordingly, no satisfactory correlation, has yet been made between the heat stability of milk and its analytical composition, nor is it possible on the basis of available data, to predict the heat stability of a concentrate from the stability of its fluid milk. Although heat treatment of fluid milk imparts stability toward clotting enzymes, as well as higher heat stability on subsequent concentration, it lowers the stability of both fluid and concentrated milks to freezing. Similarly, disodium phosphate, as a calcium sequestrant, effectively improves heat stability and is, therefore, indispensible to the manufacture of evaporated milk, but it lowers the storage stability of frozen fluid milk and HTST sterilized milk concentrates.

Increasing hydrogen ion and multivalent cation concentrations generally imply lower stability to heat, but in anomalous instances, the reverse is true. Furthermore, divalent cations lower the heat stability of concentrated milks, but markedly improve resistance to storage gelation, when added to milk concentrates after HTST sterilization.

An inocuous primary change in an otherwise stable milk colloid system may initiate a series of complex interactions that culminate in final coagulation. Thus, the absorption of approximately five per cent moisture, by a stable milk powder, initiates a phase transition of amorphous lactose to the crystalline hydrate, and a Maillard type interaction of the lactose with the casein, succeeded by degradative changes in the complex, brown pigment formation, appreciable increase in acidity and eventually, a total loss of protein solubility. Similarly, the massive crystallization of lactose in frozen milk, as a result of submicroscopic crystal nuclei generated during processing, freezing, and storage, has been shown to initiate a coagulation process ultimately involving the total caseinate dispersion. As a final example of such stability interdependence in the milk system, it is worth noting that homogenization, which is of negligible direct consequence to the caseinate micelle, nevertheless effects a substantial decrease in the stability of milk to heat, salts, alcohol, etc., through adsorption of the casein at the vastly increased fat globule—serum interface. It is therefore apparent that any appraisal of the stability of the caseinate dispersion must contend with the specific changes in the casein and serum components elicited by the various process treatments. Such irreversible changes, the most prominent of which are denaturation and hydrolytic alterations of the proteins, will now be considered in detail.

Properties of Denatured Proteins

The solubility of proteins is reduced by denaturation, a process generally regarded as any modification of the native protein structure, exclusive of primary covalent bond hydrolysis. The increased reactivity of specific groups of denatured proteins,[4,52,119,205,278] changes in the hydrodynamic properties of their solutions,[28,119] and increased accessibility to proteolytic enzymes[5,19,119,133,228] provide substantial evidence that denaturation of globular proteins constitutes an unfolding of their normally folded polypeptide chains. As a consequence of such structural changes as are incurred by denaturation, protein molecules generally acquire more hydrophobic properties,[161] evidenced by reduced solubility and heightened sensitivity to electrolytes. A slight increase in the isoelectric point[271,311] and generally small changes in the electrochemical and hydration properties of the proteins are evoked by denaturation.[131]

The large increase in specific viscosity of denatured protein solutions is generally attributed to the increased assymmetry of the unfolded molecules rather than to increased hydration or molecular aggregation.[279,356] In fact, the release of water observed in some denatured proteins by dilatometric measurement of volume contraction,[142] and the general loss of hydrophilic properties, are inconsistent with any concept of increased hydration.

An alteration of molecular weight is by no means an invariable consequence of denaturation.[278] The coagulation of denatured protein is a distinctly secondary effect[42–44] involving the formation of intermolecular linkages by groups which are displaced to the surface of the protein by the unfolding process. Interaction of denatured molecules has been variously ascribed to salt linkages between ionic groups of the protein,[87,132,262] interaction of nonpolar amino acid residues,[161,236,321,474] hydrogen bond formation,[432] and sulfide linkages.[4,99,174,191] Waugh[432] has stressed that "the randomization of molecular structure resulting from denaturation should afford a lower degree of complementation of polar groups than is possible in the native state." This is held inconsistent with the prevalent view that the characteristic insolubility of denatured proteins is derived primarily from intermolecular salt bridges between ionic groups. The significance of nonpolar group interactions of denatured proteins was advanced by Wu[474] and supported by a number of investigations.[236,321,432] Eilers[78] attributes the lower stability of denatured milk protein dispersions to the nonpolar surface groups, devoid of charge and hydration, which provide available sites for aggregation through van der Waals' attractive forces.

Casein Denaturability

The insolubility of "native" casein at the isoelectric point, its sensitivity to coagulation by electrolytes, and resistance to conventional denaturing processes, has led several investigators to regard casein as an inherently denatured protein. McMeekin[252] cited several important criteria that tend to characterize casein as having the properties of a denatured or unfolded molecule. The specific rotation of casein, $[\alpha]_D^{25}$, is $-101°$ at pH 6.9, a value that remains constant for casein isolated under neutral, acid, or alkaline conditions. The optical properties of the casein also remain unmodified by heat treatment or exposure to $5M$ guanidine hydrochloride. In contrast, McMeekin demonstrated a specific rotation increase in β-lactoglobulin solutions from $-43°$ to $-80°$ by heat denaturation at pH 8.5, and an increase to $-114°$ by guanidine hydrochloride denaturation, optical rotation changes typical of proteins subjected to denaturing treatment.[104]

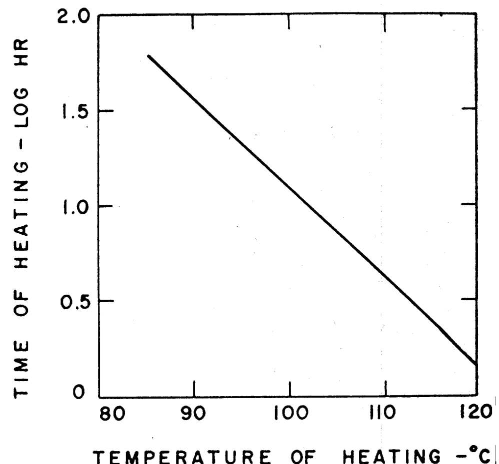
The susceptibility of casein to fairly rapid proteolytic digestion, without preliminary denaturation, further conforms to the behavior of denatured proteins.[5,228] The viscosity and streaming birefringence of its solutions indicate that casein is an elongated molecule, possessing a natural assymetry attained by other proteins upon denaturation. The resistance of casein to all attempts at crystallization has been advanced as additional evidence. Halwer,[117] in an attempt to measure the molecular weights of α and β-casein by light scattering, observed a dependence of apparent molecular weight on the electrolyte concentration of the casein solution and assessed this to be the attribute of a denatured molecule. Wright[469] inferred from the identity of racemization curves of casein from raw and autoclaved milk, that casein is essentially unaltered by normally denaturing temperatures.

Effect of Nonenzymatic Hydrolysis on the Stability of Casein

The hydrolytic cleavage of peptide and phosphate bonds in casein, resulting from enzymatic or rigorous heat treatment of milk, is not conventionally classified as a denaturation process. However, structural changes of this hydrolytic type exert such profound influence on the stability of the caseinate complex that it is essential to consider, in detail, their contribution to the coagulation of milk.

Relatively minor changes occur in the nonprotein nitrogen (NPN) during pasteurization or HTST sterilization of milk.[185,259,357,363] Belec and Jenness[13] ascertained that α-caseinate solutions heated at 135°C. hydrolyze slightly more rapidly than those of whole or β-caseinate, attaining a nitrogen release level of 5% in 20 min., and approximately 15% in 60 min., at pH 6.7. A moderate increase in the NPN fraction of HTST sterilized milk during storage has been attributed to a gradual breakdown of β-casein.[185] The increase in NPN is maximal at 37°C. Reactivation of a β-casein specific protease in milk is suggested as a possible cause for the hydrolysis noted.

Under rigorous heat treatment, casein evidences substantial hydrolysis with respect to nitrogen, and particularly phosphorus. Howat and Wright[169,171] found 15% of the nitrogen released as small fragments in neutral sodium or calcium caseinate solutions heated for five hours at 120°C., complete dephosphorylation of the sodium caseinate, and 85% dephosphorylation of the calcium caseinate system. The base binding capacity ot dephosphorylated casein is lower than that of unheated casein, at pH 6.9. This difference in base binding capacity is nearly 10^{-4} gm. equivalents of alkali per gram of protein, representing a 16% decrease in the base binding capacity of the heat dephosphorylated casein. Insolubilization of three per cent calcium caseinate solutions at pH 6.9, heated in the range 90°–115°C., is accompanied by a proportionate dephosphorylation of the casein (Fig. 60), presumptive evidence that the coagulation and phosphate release may be related reactions. The reaction velocity for both dephosphorylation and



Data from Howat and Wright[172]

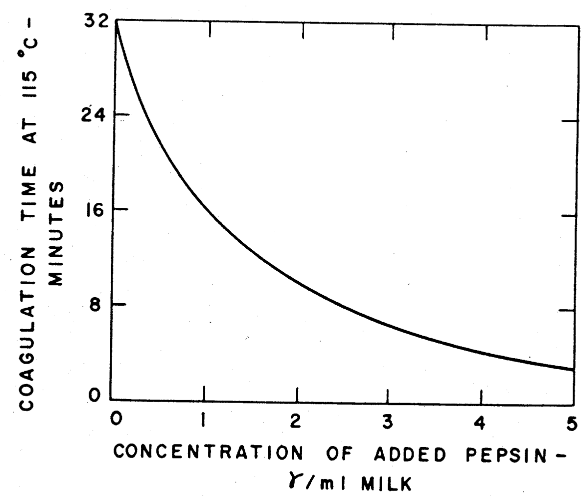
FIG. 60. TIME-TEMPERATURE RELATIONSHIP FOR COAGULATION AND DEPHOSPHORYLATION OF 3% CALCIUM CASEINATE SOLUTIONS AT pH 6.9

Line represents common points for 50% protein coagulation and 45% dephosphorylation.

coagulation increases threefold for each ten degree rise in temperature. However, phosphate does not appear to be liberated in evaporated milk aged 6–9 months at room temperature.[169] Comparable studies on phosphorus liberation from casein in milk coagulated under various time–temperature conditions, similarly led Torboli[401] to conclude that casein coagulation is related to dephosphorylation. The P/N ratio of casein isolated from milk, heated for 90 min. at 120°C. to the point of coagulation, decreases from 0.056 to 0.033.[330] Studies of casein dephosphorylation by Belec and Jenness[13] extend to conditions more nearly approximating those of normal heat process treatments. Dephosphorylation of both sodium caseinate and the casein in skimmilk, heated at 110°–140°C., conforms to first order kinetics with an energy of activation of 25–29 kcal/mol. The rate of dephosphorylation is almost identical for α- and β-caseinates and is independent of pH in the range 6.0–7.0. Twofold concentration of skimmilk increases the rate of dephosphorylation, while preheating at 90°C. for 10 min. decreases the rate by 20–25%. The observation that crystalline egg albumin acquires precipitability by multivalent cations, but loses heat coagulability when phosphorylated, constitutes an interesting analogy to the casein system.[134]

Effect of Enzymatic Hydrolysis on the Stability of Casein

Mild enzymatic proteolysis of casein renders it highly susceptible to coagulation by multivalent cations. The remarkable degree to which casein can be so sensitized is demonstrated by the fact that rennet concentrations of only 1 part in 5 million of milk will initiate clotting.[88,106,392] At subclotting enzyme levels, the effect of incipient proteolysis is still manifested by a substantial decrease in the stability of the milk to heat, freezing, and concentration as well as to the addition of salts and alcohol. Tarassuk and Nury[390] demonstrated that a marginal hydrolysis, resulting from the exposure of evaporated milk to only 0.003 ppm crystalline pepsin at 3°C. for one hour, effects a measurable reduction of the heat stability. Increasing levels of enzyme activity progressively reduce heat stability as shown in Fig. 61.



From data by Tarassuk and Nury[390]

FIG. 61. THE EFFECT OF INCIPIENT PROTEOLYSIS OF UNSTERILIZED EVAPORATED MILK ON ITS STABILITY TO HEAT

Enzyme exposure, 1 hr. at 3°C.

Incipient proteolysis, involving modification of primary structural linkages prior to generalized peptide bond hydrolysis, merits consideration as a protein denaturation process. The concept of enzymic denaturation of proteins, as the primary phase of proteolysis, was first advanced by Linderstrom-Lang *et al.*[225] The conversion of fibrinogen to fibrin, in the blood clotting process, is effected by the enzyme thrombin which has been proposed to function as a "denaturase," catalyzing the cleavage of weak bonds between folded peptide chains.[468] Similarly, the equivalence of optical rotation changes produced in native and in denatured β-lactoglobulin by trypsin and urea has been construed as further evidence that denaturation by enzymes may precede general proteolysis.[46] Numerous investigators have drawn analogies between heat and rennin coagulation of casein.[72,123,390] The heightened sensitivity of phosphorized, alcohol denatured serum globulin to calcium salts led Rimington[340] to propose that rennin action on casein may be analogous to the denaturation of globular proteins by heat or alcohol. According to Berridge[20-22] rennin may activate casein by breaking critical bonds, leaving the molecules susceptible to thermal denaturation at moderate temperatures. The high temperature coefficient for the rate of coagulation of rennin activated casein was advanced in support of this premise. The Q_{10} value for the coagulation rate process, was found to range from 13 to 16, closely approximating the uniquely high temperature coefficients for protein denaturation processes generally. Comparably high temperature coefficients were obtained by Pyne.[326]

Whether such high temperature coefficients constitute specific evidence of a true denaturation process, or merely represent a coincidence of values, is uncertain. Zittle *et al.*,[482] finding comparably high temperature coefficients for the aggregation rate process in casein generally, reject the denaturation hypothesis as applied to paracasein specifically. Coefficient values of 14 for α-casein, 19 for whole casein, and an inordinately high temperature sensitivity for β-casein, led Zittle to conclude that the temperature-calcium sensitivity of paracasein merely represents the aggregation characteristics of the normal casein complex, from which a stabilizing component has been eliminated. The generally high temperature coefficients for the casein aggregation process is compatible with the views previously described, that casein displays many of the attributes of a denatured protein. The hydrophobic properties inherent to casein, and acquired by native proteins only upon denaturation, may further relate to its unusual abundance of nonpolar, amino acid side chains.[432]

The organic phosphorus in casein, recognized as a critical site for calcium binding and interpolymer complexing, has attracted considerable interest as a pivotal factor in enzymatic coagulation. Whether hydrolysis of a critical phosphorus linkage is involved as a primary function in the enzymatic coagulation of casein, remains problematical. Available evidence indicates that the phosphorus probably occurs as a single monoester type of linkage in α, β, and whole caseins.[154,186,314] This current view is in contradistinction to earlier conclusions that the phosphorus is distributed in α-casein as phosphomonoester, mixed phosphoamide ester, and pyrophosphate ester linkages, and in β-casein as the phosphodiester, based on phosphatase enzyme specificity toward the several casein substrates.[312,313] It seems probable that the organic phosphorus in casein is bound to the hydroxyl groups of serine or threonine, since these amino acids have been identified in phosphopeptides released by hydrolysis of casein.[148,154,314] Some degree of dephosphorylation of casein may occur in milk due to the activity of milk phosphatase. The rates of dephosphorylation, of both calcium sensitive α-casein and whole casein, by native milk phosphatase at the optimum pH range of 6–7, are comparable.[476] Its capacity for dephosphorylation exceeds 80% of the available phosphorous in casein. Enzymatic dephosphorylation shifts the isoelectric point of casein toward the more alkaline side.[384]

Phosphoamidase activity, attributed to rennin, pepsin and chymotrypsin on the basis of their capacity to hydrolyse the P-N bond in N(P-chlorophenyl) amidophosphoric acid, has led to the proposal that hydrolysis of this linkage may precede general proteolysis as the primary step in the clotting process.[159,160] Although subsequent attempts to confirm phosphoamidase activity on this substrate or toward casein have proved negative,[242,313] Berridge[21] maintains that a single phosphate bond could conceivably be broken with the phosphorus atom remaining attached to casein through alternate groups. Early studies on dephosphorized casein[341] dating back to 1926, held it to be noncoagulable by rennin, but recoagulable upon rephosphorization with phosphorus oxychloride.[340] Hsu *et al.*[173] demonstrated that the rennin coagulation time of casein previously dephosphorylated with potato phosphatase increases with the level of dephosphorylation. However, uncertainty as to the organic phosphorus structure in casein, as well as to the phosphatase activity of milk clotting enzymes, renders interpretation of the primary mode of clotting action, or analogies drawn between heat and enzymatic clotting, purely speculative.

Although the specific structural bond that is altered during enzymatic clotting remains obscure, it appears that the essence of the clotting process constitutes elimination of κ-casein's micelle stabilizing capacity. The essential role of κ-casein in rennin clotting is embodied in Waugh's[434] disclosure that κ-casein is rapidly converted to the paracasein form, losing approximately 20% of its molecular weight, and attaining thereby a high sensitivity to calcium. The conversion of α_s- and β-casein to their respective paracaseins occurs only $10^{-2.5}$ and 10^{-3} times as rapidly. These relative conversion rates furthermore indicate that, in the time interval normally required for milk clotting, the κ-casein is likely to be the only component converted to a paracasein. Finally, Waugh demonstrated that the micelle stabilizing capacity of κ-casein is completely dissipated by exposure to rennin. Wake[425] advanced evidence in further support of κ-casein as the primary site of rennin action, by analysis of the nonprotein nitrogen released from purified casein components by crystalline rennin, as a function of time. No significant quantity of NPN, soluble in 12% trichloroacetic acid, is released from kappa-free casein in 20 min. Only 1% NPN is released from soluble whole casein, while the predominant amount, 6.7%, derives from κ-casein. The ratios of NPN released under identical conditions indicate the presence of approximately 15% κ-component in whole casein, a value in accord with that estimated by Waugh and von Hippel.[435] In further support of the protective colloid theory, Cerbulis and Zittle[39] disclosed a correlation between the relative calcium sensitivity of various casein fractions and the amount of soluble nitrogen and phosphorus released from them by the action of rennin.

A substantial effort has been invested in the characterization of the peptides released from casein by rennin, especially those arising from the κ-component.[101,116,182,183,239,262,287,426] Nine peptides appear in the NPN fraction of renneted casein.[286] One peptide comprising 50% of the total is nondiffusible, having an estimated molecular weight of 8,000. In consequence of its high carbohydrate content it has been termed a glycomacropeptide. It contains 11.7% nitrogen, 0.6% phosphorus, 10.9% carbohydrate, 5.1% galactose, 2.3% glucosamine, and 11.3% neuraminic acid, according to Brunner *et al.*,[29] whose results are generally comparable to those reported by Nitschmann *et al.*[282] However, a significantly higher molecular weight of 13,000 is obtained by Brunner. The aromatic amino acids, as well as cysteine, methionine, arginine, and histidine are conspicuously absent, or occur only in trace amounts. The macropeptide appears to contain eleven different amino acids, with a total of 57 estimated to comprise the molecule.[282] The glycopeptide released from κ-casein by rennin appears to be substantially identical with that derived from whole and α-casein.[182] The nonpeptide portion of the glycomacropeptide consists of phosphorus, galactosamine, galactose, and sialic acid (N-acetylneuraminic acid and N glycollyl neuraminic acid) according to Jolles *et al.*[183] The sialic acid portion apparently occupies a terminal position, since 80–90% hydrolysis is attained through the action of neuraminidase. The peptide released from κ-casein contains a glucid content of 28.2%, representing 74% of the total in whole casein. Comparative end group analysis of κ-casein and paracasein by Wake,[426] suggests that the rupture of a peptide bond may not be involved in the primary enzymatic clotting action.

In the emerging picture of the rennin clotting mechanism, the glycomacropeptide appears to endow κ-casein with unique stability attributes, by virtue of its unusually hydrophilic composition. If the stability of the caseinate dispersion in milk hinges upon the key stabilizing interactions of κ-casein, then the loss of an essential macropeptide, as the result of enzymatic hydrolysis, would constitute the principle mechanism for the clotting of milk. Incipient proteolysis of casein, insufficient to cause overt clotting, is accompanied by a loss of micellar stability under the influence of a variety of coagulating agents. Although the casein coagulated under these conditions may not exhibit the general attributes of a paracasein, it is reasonable to assume that a limited but measurable alteration of κ-casein could nevertheless be involved. Since intensive heat treatment, or prolonged storage of sterile milk may evoke a partial activation of casein, by hydrolytic changes comparable to those induced by incipient enzymatic proteolysis, a comparative study of the changes in the κ-casein in such systems appears desirable. Indeed, the likelihood of spontaneous structural changes occurring in the absence of proteolytic intervention is strengthened by evidence that casein does sustain a large increase in calcium sensitivity,[285] and an altered calcium binding capacity,[396] over protracted storage periods. Quantitative measurement of changes in the micelle stabilizing capacity of κ-casein now appears feasible, and the assessment of such changes in HTST sterilized milk concentrates, which exhibit spontaneous gelation during storage, constitutes a challenging research objective.

Serum Protein Denaturation

The denaturation of the milk serum or whey proteins profoundly modifies the course of milk coagulation and the rheological properties of the curd formed by acid or enzymes. When milk is heated to serum protein denaturing temperatures it generally acquires, in addition to a cooked flavor, an increased heat stability following concentration, a reduced colloidal stability when frozen and a resistance to clotting by rennin. Such changes are of practical significance in the production of cheese, milk powder and concentrated milk products.

The serum proteins constitute approximately 0.6% of the milk or 20% of the total milk protein. The composition of the serum protein fraction, based on electrophoretic analysis[209] is approximately 55% β-lactoglobulin, 12% α-lactalbumin, and 10% "proteose-peptone." The composition of the serum protein is subject to more marked variation than the casein, particularly in relation to the lactation cycle. In contrast to casein, the native serum proteins are soluble at their isoelectric point. When denatured, their solubility in the isoelectric region, or in salt solutions, is greatly diminished and under these conditions the serum proteins will coprecipitate with the casein.

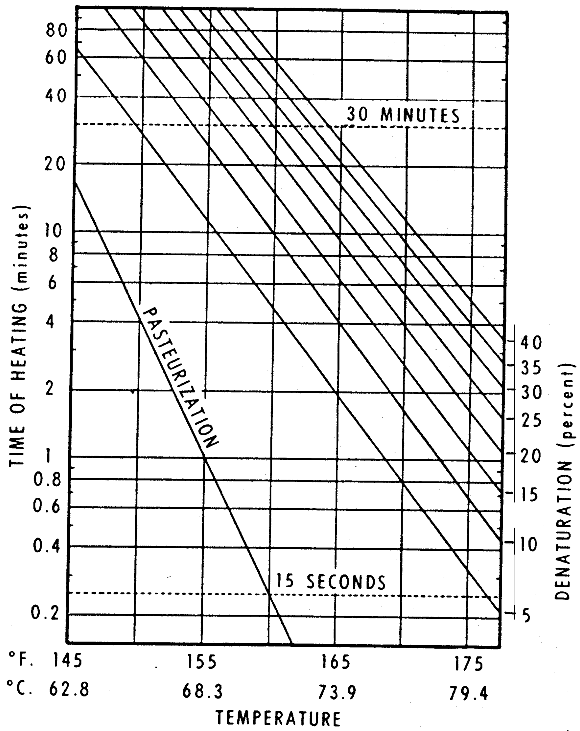
The heat stability of concentrated milk is materially increased if the fluid milk is subjected to a heat treatment before condensing. This forewarming process is essential to the stability of evaporated milk during sterilization, and much of the benefit has been ascribed to heat modification of the serum proteins,[332,344,373] as well as to favorable changes in the salt balance.[218,373] It has been generally maintained that if the serum proteins are not extensively denatured in the fluid milk, where aggregation is restricted, their subsequent denaturation during sterilization of the concentrate will culminate in extensive aggregation and reduced heat stability. Attention has also been drawn to the role of serum proteins in the heat coagulation of milk by the exceptionally low heat stability of colostral milk, which bears a high proportion of serum proteins.[71,349,373,452] However, research on the role of serum proteins in the heat coagulation of normal (noncolostral) milks, has generated conflicting evidence.[156,268,330,343,346,347,373,452] This will be considered further in the section on heat coagulation.

Kinetics of Serum Protein Denaturation

Normally, the dispersion of the serum proteins in milk is not appreciably modified by heat denaturation, as evidenced by the fact that very sizable centrifugal forces are required for their sedimentation from heated milk.[75,78,332,334] If their charge is subsequently neutralized by acidification or by the addition of salts, the denatured serum proteins will coprecipitate with the casein. However, in colostral milk coagulation may occur on heating if the globulin content is in excess of approximately one per cent, a level attained in approximately 60–84 hr. following parturition.[349]

The combined serum proteins in whey were found to be maximally heat precipitated at pH 4.5 by Okuda and Zoller,[289] and at pH 4.75–4.80 by Rowland.[351] When the denatured serum proteins in heated milk are coprecipitated with the casein by acidification to pH 4.7, a maximum of about 76% of the total soluble protein nitrogen is coagulable. Slightly less serum protein nitrogen (70%) coagulates when casein free whey at pH 4.7 is boiled.[351] The heat coagulable protein fraction of milk serum is predominantly globulin and albumin. The noncoagulable fraction has been termed "proteose-peptone." Saturation with sodium chloride generally coagulates a larger proportion of the total protein than isoelectric precipitation of heated milk,[124] probably by inclusion of a portion of the proteose-peptone fraction. Larson *et al.*[208] compared acid and salt precipitation methods and observed consistently higher salt precipitation values for serum protein denaturation in the temperature range 63°–90°C. Quantitative estimates of the total heat labile serum protein content by sodium chloride saturation therefore tend to run measurably higher. When estimated by the salt saturation method, 93–95% of the serum proteins of milk is denatured by heat treatment at 80°C. for 45 min.[124]

The loss of solubility of heat denatured serum proteins, when acidified or saturated with sodium chloride, has served as a useful criterion for kinetic studies of the overall denaturation process in milk. Early methods of analysis, based on acid precipitation, adequately demonstrated that the threshold temperature for initiation of serum protein denaturation was slightly above the general region of pasteurization conditions. For complete denaturation, heat treatments at 77.5°C. (1 hr.), 80°C. (30 min.), and 90°C. (5 min.), have been cited.[8,98,195,352] Subsequent refinements introduced in the measurement of serum protein denaturation rates enabled Rowland[349] to obtain smooth curves for serum protein denaturation as a function of temperature, with values ranging from 10.4 to 28.0% in milk heated at 63°–70°C. for 30 min. Although the energy of activation and the order of reaction could not be precisely calculated, Rowland demonstrated that the thermal coefficient of 1.5 per 1°C., in this temperature range, was of the high order of magnitude generally observed for protein denaturation. Similar time–temperature equivalents for the denaturation of the combined serum proteins in skimmilk, determined by Harland *et al.*[126] using the salt precipitation method, are shown in Fig. 62. In the temperature range 62°–

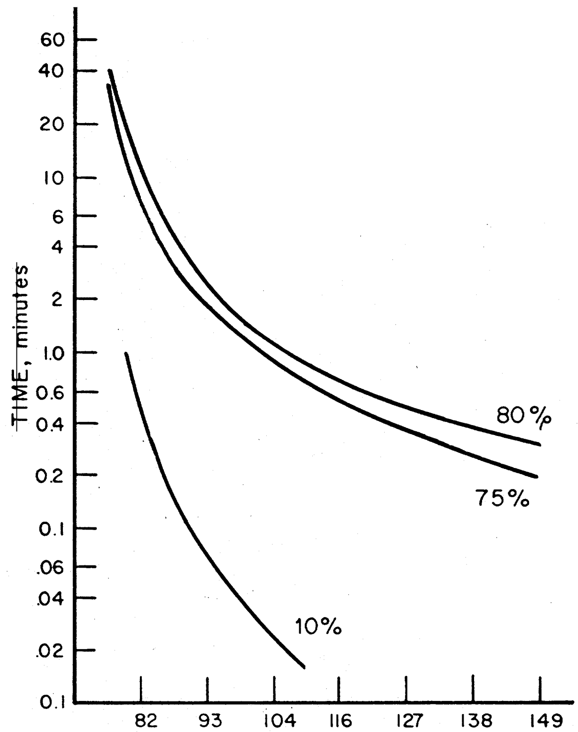


Data from Harland et al.[126]

FIG. 62. THE HEAT DENATURATION OF THE SERUM PROTEINS IN SKIMMILK

80°C. the relationship of temperature to time for a constant level of serum protein denaturation is semi-logarithmic. A temperature increase of 7.5°C. reduces the time required for a fixed level of denaturation ten fold ("Z" value of 13.5). Again heat treatments required for the pasteurization of milk are clearly below serum protein denaturing conditions.

In the high-temperature short–time range (Fig. 63), the relationship for equivalent serum protein denaturation becomes curvilinear.[128] At 80°–90°C., the Z value is approximately 19 and increases further at higher temperatures. The percentage of total serum proteins denatured is about ten per cent lower than indicated in Fig. 63, since a maximum of 90.3% of the serum proteins in the milk was found denaturable. Comparable, but somewhat higher rates of serum protein denaturation in the high-temperature short-time range were measured by Hetrick and Tracy.[141] Denaturation of albumin and globulin cannot be detected when the



From Harland et al.[128]

FIG. 63. SERUM PROTEIN DENATURATION, MEASURED BY THE HARLAND-ASHWORTH METHOD, IN SKIMMILK HEATED UNDER HIGH TEMPERATURE-SHORT TIME CONDITIONS

Fahrenheit temperatures on the original graph have been converted to the nearest centigrade value.

time–temperature conditions (HTST) for phosphatase inactivation is restricted to a minimum. The rate of serum protein denaturation varies but slightly with milk solids concentrations in the range 9–40%.[126] The first evidence of cooked flavor in milk coincides with an albumin-globulin denaturation level of approximately 58%.

Quantitative analytical methods for the direct estimation of denatured serum proteins in heated milk are lacking. Methods currently in use rely on differential determination based on the total initial serum protein content and the final residual undenatured protein following heat treatment.[124] Estimation of the heat treatment of milk products constitutes an important control in the processing of cottage cheese[252] and bread[207] where low-heat and high-heat properties are required, respectively. Excessive heat treatment of milk for use in conventional cottage cheese manufacture results in coprecipitation of the denatured serum proteins with the acid casein curd, and a loss of desirable curd tension properties.[269] Coprecipitation in cottage cheese milk offers an important potential means for increasing curd yield[81] but special care must be used in setting, cooking, and handling the curd.[409] On the other hand, milk processed for bread baking must receive a sufficiently high heat treatment to inactivate unidentified factors responsible for gluten breakdown, extreme dough extensibility, and poor loaf volume.[112,207,210] For satisfactory bread loaf volume, the milk must be subjected to a minimum heat treatment of 69°–74°C. for 30 min.[207] Milk processed for commercial bread baking is generally heated in the range of 85°–100°C.

The use of denatured serum protein values, to assess the heat treatment of milk products, is most satisfactory when the original total serum protein content is known, because the serum protein content of milk is subject to considerable variation. An extensive survey of the serum proteins in milk by Harland *et al.*[127] disclosed sizeable variations in both concentration and denaturability. The range of such variations, which appear to be related primarily to regional and breed differences rather than seasonal effects, is indicated in Table 92, reproduced from values published by Harland *et al.*[127] Wide variations were observed in the precipitability of the serum proteins in 81 milk samples heated at 74°C. for 30 min. A close correlation between the amount of protein denatured and the total serum protein content is evident. The absolute amount of serum protein denatured shows a closer correlation with the total serum protein content than do the percentage denaturation values. Such variability of denaturation rates probably reflects distribution differences in the serum proteins, as well as in the ionic composition of the milk.

The denaturation curves for the individual serum protein components, obtained by quantitative electrophoretic analysis of the serum from heated milk, place immune globulins, serum albumin, β-lactoglobulin and α-lactalbumin in order of increasing resistance to heat denaturation. The relative denaturability of the serum protein components, measured electrophoretically by Larson and Rolleri,[209] is reproduced in Fig. 64. The complete serum protein denaturation curve represents a composite of the individual serum protein denaturation curves, and the total extent of denaturation for the heat treatments indicated conforms to the values reported

TABLE 92

VARIATIONS IN THE DISTRIBUTION AND HEAT LABILITY OF THE SERUM PROTEINS IN COMMERCIAL BULKED MILK[a]

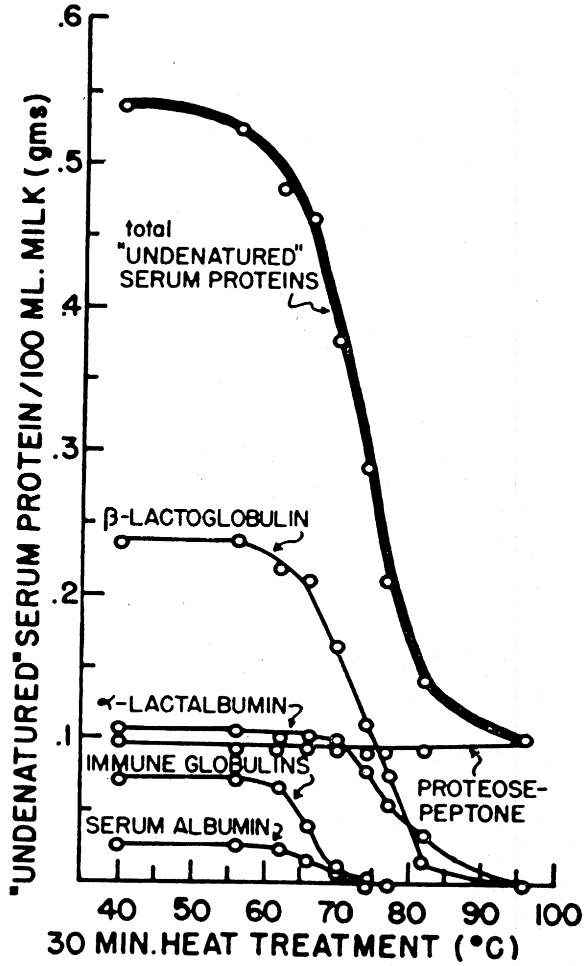
Component[b]	Range	Mean	Standard Deviation (Single Determinations)
Serum protein N (Rowland method)			
Total, mg./ml	0.82–1.48	1.04	0.12
Heat labile, mg./ml	0.60–1.10	0.76	0.08
Serum protein N (Harland Ashworth method)			
Total, mg./ml	0.62–0.91	0.76	0.06
Heat labile, mg./ml	0.55–0.85	0.66	0.06
Sulfhydryl (iodosobenzoate), meq./ml	0.19–0.44	0.30	0.16
Casein N, mg./ml	3.49–6.02	4.31	0.47
Nonprotein N, mg./ml	0.23–0.42	0.31	0.04
Total protein N, mg./ml	4.52–7.28	5.35	0.54
Total nitrogen, mg./ml	4.82–7.70	5.66	0.56
Solids nonfat, %	8.11–10.55	9.25	0.47

[a] Survey of 81 samples. From Harland, Coulter, and Jenness.[127]
[b] Data calculated on basis of skimmilk.

elsewhere.[126,207,350] Heat treatment of skimmilk at 70°C. for 30 min. denatures only 6% of the α-lactalbumin, but 32% of the β-lactoglobulin, 52% of the serum albumin, and 89% of the immune globulins, representing cumulatively, 29% of the total serum proteins. Consequently, with increasing heat treatment the composition of the undenatured proteins in milk serum will vary, increasing in the most heat resistant component, the α-lactalbumin. Over one-half the total α-lactalbumin in milk is able to withstand 77°C. for 30 min., a heat treatment sufficient to denature the serum albumin and immune globulin fractions completely.

Denaturation of β-Lactoglobulin

β-lactoglobulin, comprising 40–60% of the serum protein fraction, dominates the course of serum protein denaturation. The heat denaturation curve for the total serum protein content of milk is closely related to the denaturation curve for the β-lactoglobulin fraction.[209] Heat denaturation of β-lactoglobulin, at pH 7.0, involves at least two processes, the first of which is initiated at temperatures in excess of 65°C. and is not accompanied by electrophoretic changes.[28] However, a limited aggregation does occur, in



From electrophoretic measurements on isolated milk serum by Larson and Rolleri[209]

FIG. 64. THE DENATURATION OF THE TOTAL AND INDIVIDUAL SERUM PROTEIN COMPONENTS IN MILK HEATED AT VARIOUS TEMPERATURES FOR 30 MIN.

is primary phase of denaturation, leading to a fourfold particle ›ight increase and a higher frictional ratio. The activation energy, suming first order kinetics in the temperature range 70°–75°C., is lculated to be 48,000 calories. A secondary change, occurring at wer temperatures, leads to the formation of unlimited aggregates th an electromobility higher than that of native β-lactoglobulin. ›low 70°C., at pH 6.9, μ-0.1, this reaction is second order with an activation energy of approximately 28,000 calories. The temperature coefficient between 60° and 70°C. is 3.6, but at 75°C. the reaction is retarded, and at 99°C. entirely suppressed. This secondary aggregation process appears to be influenced markedly by the electrokinetic potential of the primary denatured protein particles.

Jenness[180] confirmed the transformation of β-lactoglobulin to a form of higher electromobility when heated alone, or in unfractionated serum protein mixture. The velocity of denaturation of β-lactoglobulin is pH dependent and is further influenced by the ionic composition of the protein solution. Alkali denaturation of β-lactoglobulin, evidenced by progressive increase in optical rotation, proceeds slowly at pH 8.25 and increases at higher pH values.[46] Based on optical rotation change, the denaturation process is unimolecular at pH 8.0–10.1, the rate being inversely proportional to the 1.1 power at 25°C. The velocity constant for denaturation at these pH values (borate buffer) is in substantial agreement with that determined by insolubility at the isoelectric point. Although heat and urea denaturation of β-lactoglobulin have been reported to be reversible,[46,225] the alkali denatured protein does not appear capable of reversion to the native form as judged by optical rotation changes, solubility, or crystallizability.[115] The rate differences noted complement the view of Briggs and Hull[28] that β-lactoglobulin denaturation involves two distinct processes. The first stage, associated with increases in optical rotation and elaboration of sulfhydryl groups is the most rapid and appears to be reversible. This process may be succeeded by irreversible changes in which the denatured protein acquires insolubility at the isoelectric point. The denaturation reaction retains first order characteristics in the pH range 5.09–8.58, at temperatures of 30°–67°C., as measured by isoelectric insolubility.[273] Groves *et al.*[115] could only partially confirm for β-lactoglobulin denaturation, the concept that protein denaturation is associated with amino group ionization.

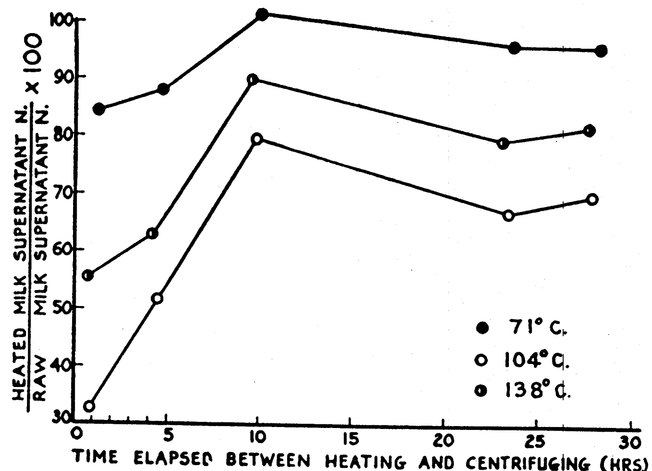
The molecular weight of β-lactoglobulin has been generally accepted as 35,000.[30,118,255,362] Light scattering and sedimentation measurements[403] indicate that the molecular unit, in the isoelectric range, is a dimer of β-lactoglobulin with fundamental units of 18,000 molecular weight. Electrophoretic inhomogeneity of β-lactoglobulin has been noted by several investigators.[288,317,399] Aschaffenburg and Drewry,[6] by working with the milk of individual cows, were able to classify the β-lactoglobulins, electrophoretically, into A, B and AB forms, demonstrating that the variations are of genetic origin. β-lactoglobulin-B exists principally in a monomeric form at pH 3.7–5.2. The observed association of β-lactoglobulin in this pH range is attributed to β-lactoglobulin-A which occurs as a polymer. The A form is the faster moving electrophoretic component at pH 4.65.[399] The β-lactoglobulin-B denatures at a considerably faster rate than the A component at 67°–75°C., in a pH 6.86 phosphate buffer, ionic strength 0.1.[107] The greater susceptibility of the B component to denaturation is evidenced with respect to liberation of sulfhydryl groups, precipitability at pH 5.0, and increased optical rotation, applied as criteria of denaturation. Energies of activation for the A and B forms of β-lactoglobulin are 65.6 and 77.1 kcal./mol, respectively. Differences in the rate of conversion of the native protein to the primary denatured forms appear to account for the observed differences in the denaturation properties of the two lactoglobulin components. Denaturation rates for the two forms in milk yield results comparable to those obtained for buffered solutions of the proteins.

An important manifestation of protein denaturation is the increased reactivity of specific groups resulting from structural modification of the protein molecule. The activation of sulfhydryl groups is one of the most readily detectable of the chemical changes accompanying denaturation.[4,278,320] A number of reagents, including *o*-iodosobenzoate, thiamine disulfide, iodoacetate and *p*-chloromercuribenzoate etc., have been introduced for the quantitative estimation of sulfhydryl (—SH) activity in denatured protein. Their application to studies on the milk serum proteins has been surveyed by Jenness[180,181] and Coulter *et al.*[51] β-lactoglobulin, the dominant sulfhydryl bearing component of the serum proteins[176,205] possesses an estimated four —SH groups per mole on the basis of a cystine equivalent of 1.3%, and a molecular weight of 36,000. Sulfhydryl group activation by heat has been associated with the origin of cooked flavor as well as the antioxygenic properties of heated milk.[50,125,176,483]

Larson and Jenness[206] applied the *o*-iodosobenzoate method to a kinetic study of the heat activation of the sulfhydryl groups in crystalline β-lactoglobulin. In the temperature range 64°–75°C., —SH groups are activated according to a first order reaction with an activation energy of 80,000 cal./mol. This activation of sulfhydryl groups may parallel the primary denaturation phase,[28] but insufficient data are available for confirmation of this relationship. There is a general correlation of sulfhydryl activation and denaturation as measured by solubility loss of the serum proteins in heated milk.[207]

The aggregation and precipitation of heat denatured β-lactoglobulin, in the presence of calcium ions, is highly pH dependent. Zittle *et al.*[477,480] observed a progressive decrease in the calcium ion concentration required to precipitate heated β-lactoglobulin as the pH of its solution is lowered. The interaction of calcium at negatively charged carboxyl sites on the protein molecule may reduce the net charge to zero and render the protein isoelectric, with consequent insolubility. In support of this view, Zittle observes that β-lactoglobulin treated with formaldehyde or nitrous acid shows greater tolerance for calcium than the unmodified protein. The loss of positively charged amino groups through such chemical modification results in an increase of both the net negative charge and the requisite calcium concentration for precipitation. The precipitation of 0.9% β-lactoglobulin, in calcium solutions heated at 90°C. for 30 min., occurs at 0.0018, 0.0027, and 0.0031 moles calcium chloride/liter at pH values of 6.7, 7.4, and 8.2, respectively. The calcium precipitation threshold is less than doubled at a 1.8% protein level. Although the calcium ion concentration in milk is normally adequate to accommodate the precipitation requirements of the β-lactoglobulin, the denatured serum proteins in heated milk do not coagulate to any appreciable extent. This is generally construed as evidence of a heat induced interaction between lactoglobulin and casein. Attempts to determine the effect of heat on the dispersion of the serum proteins in milk have been thwarted by the analytical difficulties involved in differentiating denatured serum proteins from the casein with which they tend to co-migrate electrophoretically, and coprecipitate isoelectrically. Phosphorus and sulfur partition studies of heated lactoglobulin-casein mixtures,[55] and milk,[78] failed to disclose any significant complex formation. Ultramicroscopic examinations of rennet whey also suggest that the number of ultra-microns is not increased appreciably on total denaturation of the serum proteins.[78] Identical conclusions concerning the dispersion of serum proteins have been inferred from ultracentrifugal nitrogen sedimentation analyses of heated milk (100°C., for 10 min.), at 28,800 × g.[369] However, Edmondson and Tarassuk[75] maintained that in order to discern any cosedimentation of the denatured serum proteins with casein, the centrifugation conditions must effect nitrogen sedimentation in excess of the total casein nitrogen content. On this basis, it was demonstrated that at least 33% of the denatured serum protein nitrogen in heated centrifugal whey (88°C. for 15 min.) could be sedimented at a force of 26,000 × g in 30 min. It has also been observed that nitroprusside reactive sulfhydryls, associated with the casein in heated milk are supercentrifuged in proportion to the casein sedimented.[305]

Unequivocal evidence of altered dispersion of the serum proteins in heated milk, has been provided by the experimental investigations of Sullivan *et al.*[383] The supernatant nitrogen in centrifuged milks was observed to decrease sharply in milks heated at 71°–137°C. and centrifuged immediately at 113,000 × g for 2 hr. (Fig. 65). Under



Data from Sullivan et al.[383]

FIG. 65. VARIATION OF SUPERNATANT NITROGEN IN HEATED SKIMMILK CENTRIFUGED IMMEDIATELY AND AFTER COOL-AGING AT 3°C.

Average centrifugal force, 113,000 × g for 2 hr. Milk was tubular heated for 1 min. hold time at the designated temperatures.

these conditions substantially all the centrifugable casein is removed. Heat treatment at 104°C. effects a maximum change. A 33% reduction of supernatant nitrogen under these conditions is equivalent to centrifugation of 92% of the total nitrogen in the milk. On cool aging after heat treatment, rapid equilibria shifts tend to restore the supernatant nitrogen to an intermediate value. This "hysteresis" effect appears to level off in approximately 24 hr. In a correlated experiment, 62% of S^{35} labeled whey proteins, incorporated in milk prior to denaturing heat treatment, was recovered in the ultracentrifugal sediment, which contained only 50% of the casein. Whether the denatured serum proteins sediment more rapidly through intermediate aggregation, or interaction with the caseinate complex, was not resolved.

Additional evidence of milk protein interaction has been derived from electrophoretic studies of heated milk and model protein systems.[89,250,274,366,400,406] Although interaction of β-lactoglobulin and casein is negligible in normal skimmilk,[366] Fox *et al.*[93,95] observed a distinct association of the two proteins in reconstituted commercial skimmilk powder, examined electrophoretically in the pH range 5.5–8.5 at $\Gamma/2 = 0.02$ and 0.1. β-lactoglobulin tends to migrate with the α-casein component when heat denatured, forming a complex which cannot be resolved electrophoretically at either pH 6.86 or 2.45.[250,406] The complex is of lower electromobility than that of unheated or heated β-lactoglobulin, or unheated α-casein. Electrophoretically, the tendency for the α-casein peak to increase at the apparent expense of the lactoglobulin component in heated milk, similarly implies complex formation.[366,400,406] The addition of a sulfhydryl blocking reagent, such as *p*-chloromercuribenzoate, to skimmilk before heat treatment prevents changes in the electrophoretic patterns normally associated with heat induced complex formation between the α-casein and β-lactoglobulin.[406] The rennin clotting rate of milk is suppressed by heat treatment due to a modification of the ionic equilibrium[319,324] and formation of a complex between β-lactoglobulin and casein. Moir[264,265] demonstrated a progressive decrease in the serum protein nitrogen of rennet whey from milks pasteurized at increasing temperatures. The correlated increase in rennin time was attributed to a "precipitation" of the denatured serum proteins on the caseinate micelles, restricting the access of rennin enzyme. Involvement of the milk serum proteins in the prolongation of rennin clotting of heated milk has been substantiated by Kannan and Jenness.[187] The rennin clot time of the calcium caseinate complex separated from milk is considerably prolonged if the complex is heated at 85°C. for 36 min. in the presence of added β-lactoglobulin. The clot time is further lengthened as the β-lactoglobulin concentrations are raised. Separate heat treatment of the individual components does not modify the rennin response of the caseinate complex, and serum protein constituents other than the β-lactoglobulin do not evidence this interaction.

A specific interaction between heated κ-casein and β-lactoglobulin, disclosed by Long,[234] may have important bearing on the mechanism of the forewarming process, and on the suppression of rennin clotting of heated milk. In 1:1 mixtures of these proteins in solution at pH 6.5, interaction, as evidenced by electrophoretic pattern changes, is initiated at approximately 65°C., increasing to 15% at 70°C., attaining a maximum of 83% at 85°C., and declining slightly to 76% at 99°C. Values for percentage interaction refer to globulin complexed after 20 min. heat treatments. Interaction with κ-casein increases at higher ratios of β-lactoglobulin, approaching a maximum of 2.2 gm. bound per gram of κ-casein. Interestingly, the two components interact when heated separately, though to a lesser degree. Denaturation of the β-lactoglobulin appears essential to the formation of the complex. Unheated κ-casein can, however, complex with denatured β-lactoglobulin. Fox[93] observed similar evidence of an interaction above 66°C. between β-lactoglobulin and a calcium soluble casein fraction, presumably containing κ-casein. Whether this interaction of β-lactoglobulin with κ-casein constitutes the predominant reaction observed with the α-casein complex, has not been resolved. That an interaction with the key micelle stabilizing component can occur so readily, at temperatures widely employed in various milk processes, appears highly significant and merits further intensive study.

The occurrence of additional interactions among the several serum proteins has not been adequately explored. Jenness[180] noted a tendency for the various serum protein components to migrate electrophoretically as a single peak following heat treatment, suggesting the probability of such interactions. Interspecies complex formation between such protein pairs as casein and horse serum immune globulin,[178,202] and β-lactoglobulin and serum albumin,[99] further attest to the remarkable capacity of the milk proteins for a multiplicity of interactions.

HEAT COAGULATION OF FLUID MILKS

Mechanism of Heat Coagulation

The serious voids that persist in our knowledge of the equilibria and compositional changes in milk, at coagulating temperatures, have thwarted the development of a unified theory of heat coagulation. The mechanism of heat coagulation is further obscured by the inconsistent variations in composition and heat stability of different milks, and even of milks drawn from different quarters of the udder of a single cow.[17] Consequently, no satisfactory correlations have as yet been established between heat stability and the analytical composition of normal fluid milks or their concentrates.[158,344,452]

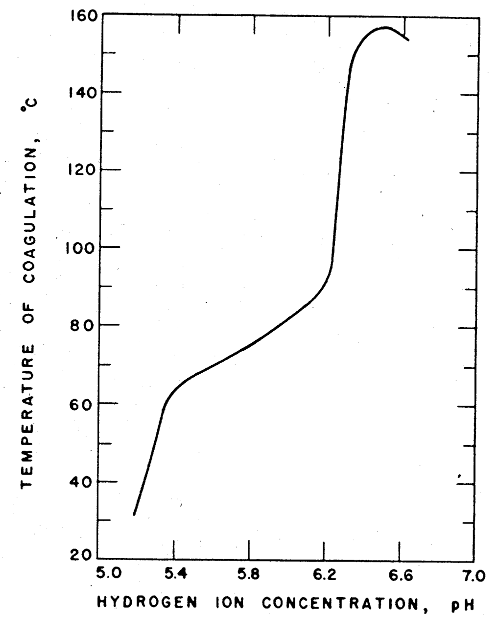
In the strictest sense, casein is not intrinsically a heat coagulable protein. Its dispersion in normal fluid milk is highly stable to heat, and may resist coagulation for upward of 12 hr. at boiling temperatures, and 1 hr. at 130°C. The heat coagulation of casein, in milks of normal stability, occurs largely as a result of compositional changes in the milk, incurred by sustained exposure to high temperatures. Of predominant significance, among the effects wrought by heat, are the increase of acidity, the conversion of soluble calcium and phosphates to colloidal form, and the induction of interactions, denaturation, and hydrolytic changes in the protein components. The impact of such heat effects on colloidal stability is further amplified in concentrated milks, whose rate of coagulation tends to increase logarithmically with milk solids concentration.[157,445]

Salt balance and acidity are generally regarded as two of the most decisive factors in the heat stability of milk. The low heat stability of colostral milks is ascribed to a higher ionic calcium level[353,452] and excessive serum protein concentration.[71,295,349,373,452] Noncolostral milks of inordinately low heat stability. (Utrecht abnormality) are of normal composition with respect to total calcium and acidity, but evidence a notably high ionic calcium activity.[360] Whereas the calcium ion concentrations in milks of normal heat stability range from 2.0 to 3.6 mM/liter of ultrafiltrate as assayed by the murexide method,[367A] ionic calcium values in milks exhibiting the Utrecht abnormality range from 4 to 7 mM/liter.[26] This defect can be simulated in milks of normal heat stability by the addition of sufficient calcium salt to elevate the ionic calcium concentration above 4 mM/liter.[367] Alkaline pH adjustment, or addition of calcium sequestrants constitute effective corrective measures for this heat stability defect.[360,361]

Pyne[325] effectively demonstrated that normal variations in the heat stability of milks relate predominantly to differences in the composition of their diffusible serum components. This was accomplished by dialysis of a series of milk samples, of graded heat stabilities ranging from 22 to 58 min. at 130°C., against a large volume of the equally pooled milks, which resulted in a leveling of their heat stabilities to a common value of 38 min. The "effective calcium ion concentration" as measured by a renneting technique,[327,330] was held to bear an inverse relationship to heat stability. However, White and Davies,[452] surveying over 100 samples of skimmilk in an attempted correlation of chemical composition and heat stability at various stages of lactation, found no adequate analytical correlation for individual milk samples. Comparison of average compositional values for grouped milk samples disclosed a tendency toward low heat stability in early lactation milks, associated with a high average composition of albumin and globulin as well as soluble and ionized calcium. Their correlations for mid and late lactation milks proved more obscure. However, susceptibility to heat coagulation at all stages of lactation tended to increase with higher levels of ionized or soluble calcium, for milks bearing low ratios of colloidal calcium to casein.

The role of colloidal calcium in the heat stability of milk has evoked similarly conflicting evidence. It has been inferred that the presence of "precipitated" calcium phosphate is likely to render milk unstable to heat,[261,372] and that the coagulation reaction involves the transfer of calcium from the protein to triphosphate.[227] A distinct endothermic change, coincident to heat coagulation, has been cited as evidence of precipitation of citrate and phosphate salts of calcium and magnesium.[217,218] The rate and extent of such salt precipitation, deduced from heat absorption measurements, are depressed in milks of higher heat stability. Pyne attributes to the colloidal calcium phosphate a significant role in heat coagulation[329,330] as well as in rennin coagulation,[324] having observed that the heat stability of milks, depleted of nearly all their colloidal calcium phosphate, is almost doubled at 120°C.[330] Nevertheless, attempts to correlate the colloidal phosphate content of milk samples with their heat stability values have persistently proved unsuccessful.[345,453] Furthermore, Zittle *et al.*[479] find negligible coprecipitation of calcium phosphate with the calcium caseinate in heated model systems, and note that the addition of freshly precipitated tricalcium phosphate is without significant effect on the coagulation of calcium caseinate. They conclude accordingly that pH and available calcium, rather than complexed phosphate, are the dominant factors in the stability of the calcium caseinate complex.

The hydrogen ion concentration of milk exerts a most profound influence on heat stability, but again the relationship is a highly complex one. The titratable acidity value or pH of milk affords a generally inadequate basis for projecting its susceptibility to heat coagulation.[260,344,372,453] The general relationship between adjusted pH and spontaneous coagulation temperatures of raw skimmilk, as defined by Miller and Sommer[260] is shown in Fig. 66. A high order of sensitivity to pH is evident for values between 6.2 and 6.4. At pH values below 6.4, added phosphate tends to displace the curve toward higher stability, while calcium has the reverse effect. Such heat stability curves have been found to exhibit a maximum, at a pH value that appears to be a specific characteristic for each milk.[18,333,345,346,373,438] Refined measurements of the pH—heat stability curves by Rose[345,346] locate the maxima for all samples within



From data by Miller and Sommer[260]

FIG. 66. THE EFFECT OF pH ON THE COAGULATION TEMPERATURE OF SKIMMILK

the pH range 6.5–6.7. These values decline to a minimum in the pH range 6.7–6.9 and with further increases in pH the heat stabilities increase substantially. The pH of maximum heat stability was found to vary between milk samples, and to fall above or below the normal pH of the milk. Rose[345] suggested that the heat stability of milk may be significantly related to the difference between its normal pH and its pH of maximum stability. The heat stability-pH curve appears to be significantly altered by β-lactoglobulin but not by α-lactalbumin or euglobulin-pseudoglobulin mixtures.[346]

The renowned salt balance theory, formulated by Sommer and Hart in 1919,[372,373] conceived the heat stability of milk as a function of the ratio of the concentration of calcium and magnesium ions to those of phosphate and citrate. The use of stabilizing salts to enhance the heat stability of milk proved, as an outgrowth of this concept, to be of inestimable value to the evaporated milk industry. However, no rigorous experimental correlation has, as yet, been made between the heat stability of milks and their concentration of individual ionic species or of their ionic ratios. Discerning measurements by Rose[345,346] indicate that the effect of calcium, phosphate, and citrate additives on heat stability relates, to a considerable extent, to the resulting minor changes in the pH of the milk.

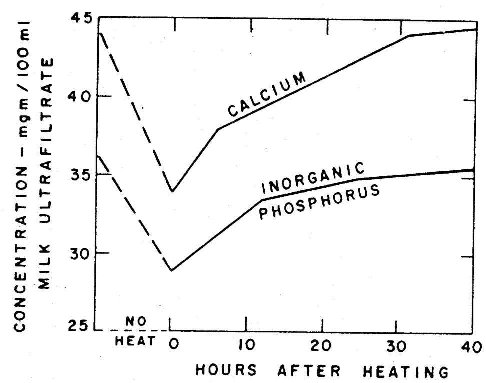
The development of a unified theory of heat coagulation has yet to be realized, but very substantial progress has been made toward defining some of the highly complex chemical changes in milk that are elicited by coagulating heat treatments. The effect of heat on milk is an essential component of the coagulation problem and will therefore be considered at length in the following section.

Effect of Heat on Calcium and Phosphate in Milk

The soluble calcium and phosphate content of milk is reduced by heat, an effect first noted by Söldner[368] and since confirmed and elaborated on by numerous [45,60,114,144,217,218,227,238,243,261,410] investigators.

The soluble (diffusible) calcium has been estimated by a variety of methods of phase separation, including renneting, ultrafiltration, and dialysis. The total calcium concentration is approximately 30 mM per liter of which 10 mM is diffusible and 2–3 mM is ionized.[45,144,361,367,411,412,419] Calculating from the dissociation constants of calcium citrate and calcium phosphate, Smeets[367] verified the average ionic calcium concentration of 2.75 mM per liter, as determined by the murexide method. About 35% of the soluble calcium in milk is in the ionic state, while 55% is complexed to citrate and 10% to phosphate. Christianson[45] measured 2.0–2.3 mM calcium and 0.82–0.85 mM magnesium per liter of milk at 25°C. by an equilibrium exchange resin method. Both the total soluble and ionic calcium concentration are reduced by heat treatment. Under pasteurizing conditions, the reduction of soluble calcium and phosphorus concentration is slight,[14,144,243,352,394] but significant losses of soluble calcium and phosphorus are sustained above 75°C., relative to the temperature and duration of heat.[14,144,238,348,394] Values reported for salt distribution changes in heated milk show large discrepancies which may be attributable to inadequate precautions against equilibrium shifts in the analytical procedures employed, and to variations in the time elapsed between the heat treatment and analysis.

Jenness[144,179] observed an initial loss of approximately 25% of the soluble calcium in milk heated at 78°C. for 30 min., and cooled before analysis. On aging at 5°C. a gradual reversion toward the original soluble calcium level occurs over a period of 24–48 hr. (Fig. 67). The soluble phosphorus undergoes a similar change in heated milk. The decrease in soluble calcium and phosphorus im-



From data by Hilgeman and Jenness[144]

FIG. 67. ULTRAFILTERABLE CALCIUM AND PHOSPHORUS IN MILK HEATED AT 78°C. FOR 30 MIN., MEASURED INITIALLY AND AFTER COOL-AGING AT 5°C.

mediately after heat treatment, and its reversion on cool aging, has been confirmed by analysis of calcium and phosphorus in centrifuged milk.[76] Although substantial reduction of ionic calcium in milk heated at 85°–120°C. has been found, agreement is lacking on the reversibility with aging.[9,45]

Van Kreveld and Van Minnen,[411,412] using an ion exchange equilibrium method, found an average of 2.2 mM ionic calcium and 0.8 mM ionic magnesium per liter of bulk raw milk, with little indication of seasonal variation. The calcium ion concentration is reduced from 2.0 mM per liter in a raw milk sample, to 1.85, 1.65, 1.50, and 1.40 when heated for 1, 3, 10, and 30 min., respectively, at 95°C. The effective magnesium ion concentration decreases progressively from 0.8 mM in the raw milk, to 0.75, 0.60, 0.55, and 0.45 mM, for the heat treatments cited. The concentration of both divalent cations is increased in evaporated milk, but the increment is less than that for potassium and sodium, implying precipitation of some of the calcium and magnesium, possibly as the phosphate salts. This relative departure from the anticipated activity of calcium and magnesium ion becomes more pronounced with increasing milk solids concentrations. The combined effect of disodium phosphate stabilizer and heat sterilization may lower the ionic calcium concentration in evaporated milk 20–40%. The addition of 0.15% disodium phosphate to raw skimmilk lowers the serum calcium/total calcium ratio from 31.7 to 21.6%, which is further reduced to 11.6% following heat treatment at 88°C. for 15 min.[76]

Since changes in the electrolyte composition of heated milk are conceivably involved in the mechanism of heat coagulation, its measurement at elevated temperatures constitutes a critical study. This is particularly so in view of the evidence that heat induced changes in the salt balance revert on cooling and may escape detection. Analyses by Rose,[348] of ultrafiltrates separated from milk samples heated at 94°C., establish that the changes in the salt balance are more extensive than had been anticipated.[348] Ultrafilterable total calcium and phosphorus, separated from milk at 94°C., is reduced by as much as 50 and 18% respectively over raw milk controls, while calcium ion concentration sustains a 60% reduction. These changes, a function of temperature level, generally attain equilibrium within five minutes. Reversion of the salt balance to within 75–90% of the value for the original unheated milk occurs on cool aging at 5°C. for 22 hr. Identical measurements on ultrafiltrates of calcium phosphate solutions, and artificial sera, indicate that the changes in heated milk evolve primarily from the solubility properties of calcium phosphate, and not from a modified dissociation of calcium caseinate or calcium citrate. Since the calcium ion concentration decreases sharply at higher temperatures, Rose *et al.*[348] reject the assumption that heat coagulation may derive from increasing calcium ion activity, due to any assumed higher dissociation of calcium citrate,[82] or increasing acidity at elevated temperatures.[330] The heat stability of milk bears no apparent correlation with the composition of its high temperature ultrafiltrate, indicating that heat derived changes in the salt balance may not be the predominant factor in heat coagulation, as generally assumed.

Partial reduction of the soluble and ionic calcium concentration in heated milk is attributed to a conversion of soluble calcium phosphate to the colloidal state. The partially reversible heat displacement of the equilibrium between calcium phosphate in solution, and that associated with the colloidal phase, appears to be involved in the rennin hysteresis effect.[294,318,319,324] The state of the colloidal calcium phosphate and its association with the caseinate complex has evoked considerable dispute,[393] having been described variously as tricalcium phosphate,[57,77,323,335,368] dicalcium phosphate[413] or a mixture of several phosphates.[84,331] Van der Burg[31] proposed that the calcium and phosphate may be separately linked to the casein as discrete groups bound to the charged sites on the protein. The constancy of the Ca/P ratio tends to invalidate the concept that the calcium and phosphate is independently adsorbed in a random composition at the basic and acidic sites on the casein.[76,332] The distribution of the colloidal phosphate as a function of casein micelle size has been the subject of similar dispute. Opposing conclusions hold that the colloidal phosphate is uniformly distributed,[57,76,91] and that the larger micelles are richer in colloidal phosphate.[165,332] Pyne and Ryan,[331] on the basis of a modified oxalate titration for the analysis of the phosphate salt composition, defined the colloidal phosphate as 88% tricalcium, with the residual 12% probably in the dicalcium form.

Evenhuis and de Vries[83,85] suggest that recrystallization of the calcium phosphate to hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ occurs in heated milk, a process that is greatly accelerated if a large surface area for nucleation is provided, as in the form of inactive yeast cells.[31] This precipitation and recrystallization process begins at a temperature of 60°C., increasing rapidly at higher temperatures. Assuming the phosphate in milk to be recrystallized to hydroxyapatite on heating, the Ca/P ratio of the precipitated phosphate would be 1.67, a value in agreement with the analysis by van der Burg.[31] Approximately 48% of the casein bound calcium and magnesium is precipitated with phosphate, in milk treated with yeast cells for 20 min. at 120°C. The calcium in raw rennet whey is readily precipitated with the denatured serum proteins and does not require the intervention of the foreign yeast cell surface. According to Evenhuis and de Vries, 10.8, 18.6, 28.3, and 30.0% of the calcium phosphate in rennet whey is precipitated at 60°, 80°, 100°, and 120°C. respectively, within 5 min. of heating.[85] The precipitated calcium phosphate is highly resistant to re-solution on cooling. It is suggested that precipitation of soluble calcium as the apatite crystal, presumed to form in heated milk, would leave the milk unsaturated with respect to calcium and thereby enhance its heat stability. The beneficial effects of forewarming have similarly been related to heat modification of the salt equilibrium. In agreement with the observations of others,[31,199] Pyne[330] suggests that the benefit of strong preheat may originate with detachment of the colloidal phosphate from its complex with the caseinate, a change comparable to that noted in the polarograms of barium caseinate–barium phosphate complexes.[325]

The Effect of Heat on the Acidity of Milk

The acidity of milk increases with temperature,[66,260] partially as a result of probable changes in the buffer capacity of the milk salts.[260] Miller and Sommer[260] observed that the pH of skimmilk decreases approximately 0.1 pH for each 10°C. temperature rise, a change that is enhanced by added calcium and opposed by phosphate. Rose *et al.*[348] report the hydrogen ion concentration of a 94°C. milk ultrafiltrate to be at least twice that of a comparable 25°C. ultrafiltrate. Concentration of milk effects a significant pH decrease which may contribute to the greater heat susceptibility of condensed milks.[78,170,372] Under prolonged heat treatment at elevated temperatures, additional acidity is developed due to secondary changes in the milk. Acidity may be derived, under these conditions, from thermal decomposition of the lactose to volatile acids, interaction of lactose with the milk proteins, casein ester phosphate cleavage, and displacement of the calcium phosphate equilibrium.

Of the total acidity developed in milk, heated at 120°C. for 90 min. to the point of coagulation, (pH 5.87), Pyne and McHenry[330] attribute one half to lactose decomposition, one third to casein ester phosphate cleavage and the residual acidity to a displacement of phosphate equilibria. Such heat-developed acidity contributes materially to the heat coagulation of milk.[327,330] In a study of 26 samples of fluid skimmilk it was noted that the pH at the time of coagulation ranged from 5.5 to 6.0, the more rapidly coagulating samples generally developing the least acidity.[330] The degree of acidity developed, before heated milk coagulates, appears to be inversely related to the calcium ion activity, suggesting that developed acidity may supplement the coagulating action of ionic calcium. On the average, fluid skimmilks with an "effective calcium ion concentraton" in excess of 4.8 mM per liter, coagulated within 20 min. at 130°C.[330] At coagulation, the pH had been depressed only 0.4 units. Milks containing 4 mM effective calcium ion per liter, survived 30 min. at 130°C. at which time the pH had declined 0.7 units. At a calcium ion concentration of 3.7 mM per liter, the average milk withstood coagulation at 130°C. for periods up to 60 min., sustaining a pH decrease of 1.0 unit. The higher calcium activity presumed to develop at the lower pH values of heated milk has been advanced as a supplemental factor in the coagulating action of heat derived acidity, but Rose *et al.*[348] disproved this assumption by direct analysis of the ultrafiltrate separated from milk heated at temperatures above 94°C.

Acidity Derived from Heat Decomposition of Lactose

Thermal decomposition of lactose, as a partial source of acidity in heated milk, has been well documented.[108–110,194,199,266,267,330,455,456] The rate of acid formation has been found proportional to the lactose concentration, and the formation of formic and lactic acids in heated milk has been correlated with the degree of lactose decomposition.[199] Formic acid constitutes approximately 57% of the acid generated in milk heated at 116°C. for 2 hr.[108,109] and 75% at 100°C. for 6 hr.[267] The lactic acid produced in milk heated sufficiently to decompose 25% of the lactose, accounts for less than 5% of the total acidity.[108] Whey heated at 116°C. for 2.5 hr. shows only 11% of the titratable acidity increase produced in comparably heated skimmilk, although a similar degree of lactose decomposition is sustained.

In addition to formic and lactic acids, butyric, propionic, acetic and pyruvic acids have been identified chromatographically in heated milk.[194,266,267] The total acidity generated in a typical sample of skimmilk, heated at 100°C. is 78, 133, 288, and 560 microequivalents per 100 ml at 1, 1.5, 2, and 6 hr. intervals. The heat-derived acidity may vary considerably with different milk samples. The total acidity values in skimmilk samples, heated at 100°C. for 6 hr., range from 361 to 588 μ eq per 100 ml, comprising 314–468 of formic acid, 56–153 of acetic eq, 2–40 of lactic acid, and 4–12 of pyruvic acid.[267] Butyric acid probably originates with fat hydrolysis, while lactose decomposition or oxidative deamination of alanine are proposed as possible sources of pyruvic acid in heated milk.[300]

The presence of stabilizing salts appreciably increases the rate of acid formation during heating.[108,266,267] The addition of 0.5% disodium phosphate may triple total acid formation primarily through an increase in the formic, lactic, and acetic acid components.

Acidity gradually develops during prolonged storage of evaporated milk and dried milk products at moderate temperatures, as a further consequence of sugar decomposition reactions.[50,51,65,136,299] At 38°C. storage, 50–75% of the titratable acidity increase in evaporated milk is due to formic acid. Lactic acid formation during storage is negligible.

Acidity Derived from Lactose-Milk Protein Interaction

The development of acidity in heated or aged sterile milk is accompanied by a brown discoloration, attributed to the formation of a poorly defined class of high molecular weight pigments termed melanoidins.[51,108,113,135,136,299,300] The weight of experimental evidence supports the view that this browning reaction evolves from a Maillard type condensation of lactose with the milk proteins,[96,135,136,212,213,215,222,270,296,297,299,301,303,307] rather than a caramelization of the lactose.[188,199] Orla-Jensen and Plattner[290] initially observed that neither casein nor lactose solutions browned when heated separately and proposed that an interaction is responsible for color formation in heated mixtures. Ramsey *et al.*[336] postulated that a Maillard interaction, between the aldose group of the lactose with the free amino groups of casein, is the primary source of color in heated milk. Patton and Flipse[301] isolated a colorless, labile complex of lactose and casein capable of browning when exposed to heat. The browning of fluid milk, and acid development from thermal decomposition of lactose, are not significant below 80°C. under the usual pH conditions of milk,[32,113] although sugar-protein complexing can be detected.[111,113,301] With increasing concentration, temperature, and pH, the rate of browning and acidity formation is markedly enhanced.[32,51,136,299,364] In the 95°–120°C. temperature range, the Q_{10} for the rate of browning, as measured by reflectance changes, is 3.1.[32]

The occurrence of an interaction between lactose and the proteins in milk has been established on the basis of radioisotope tracer studies,[301,302] and changes in the concentration of free amino nitrogen and reducing sugar.[96,121,129,135,136,212,213,215,222] Patton and Flipse[302] concluded, from the distribution of C^{14} in milk heated with added lactose-1-C^{14}, that the formation of formic acid and maltol from lactose involves carbon atom No. 1. The glucose moiety appears to be the primary source of sugar decomposition in heated milk. The primary sites of interaction on the protein have been identified as the un-ionized amino groups on the side chains of the diamino acids, or the terminal alpha amino groups of the proteins.[121,135,136,212,213,215,245,263,270,303] Approximately 90% of the total free amino groups are the ϵ-amino groups of the lysine residues. Casein,[135,136,301] β-lactoglobulin,[96,129] and minor nitrogenous components of milk[1] may contribute supplemental reaction sites, but the casein plays a dominant role in the browning of milk.[108,136,300,301]

The acidity derived from interaction of lactose with the proteins in heated milk may originate from two related sources. Initially, the acidic character of the protein is progressively enhanced as the basic amino groups are bound through interaction with lactose.[113,189,190,263] The lactose-protein complex is unstable, and secondary fragmentation of the sugar to various acidic decomposition products provides a significant additional source of acidity.[266,267,300] The mechanism of acid formation through sugar-protein interaction has not been adequately resolved. Lactose reacts with the available amino groups in a 1:1 ratio[130,136] forming heat-labile, colorless complexes.[111,130,301] When subjected to heat, the bound lactose undergoes fragmentation and ultimately yields the several acids identified in heated milk. Numerous other compounds including 5-hydroxymethyl furfural,[298] furfuryl alcohol,[304] maltol,[296] lactulose,[2] acetol and methyl glyoxal,[192] some of which may serve as browning intermediates, have also been identified. Keeney *et al.*[192] propose that the formation of formic, lactic, and acetic acids from lactose in heated milk may involve methyl glyoxal as an intermediate. The decomposition of the lactose appears to involve a catalytic fragmentation of the sugar by the ϵ-amino groups of the lysine contained in casein.[122,149,214,300,301,306] A probable route for the fragmentation of lactose complexed to casein, suggested by the work of Hodge *et al.*[149,150] involves an amadori rearrangement, i.e., an isomerization of the N-substituted lactosylamine to a 1-amino-1-deoxy-2-ketose. Such rearrangement has been implicated in the browning of model aldose–amine systems. The carbon bonds α and β to the carbonyl group become weakened as a result of this isomerization, leading to fragmentation by dealdolization of the ketose.

The formation of significant concentrations of acid in heated protein-free, buffered lactose solution, is ascribed to caramelization of the lactose.[188,199,290,416,436,455,456] Caramelization is accelerated by phosphates and citrates. Furthermore, under proper pH and buffer conditions, lactose solutions heated in the absence of amino compounds will yield many of the same carbohydrate fragmentation products identified in heated sugar-protein systems.[298–300] The amino groups of the protein have been viewed, therefore, as alkaline catalysts in the decomposition of lactose.[300,306] Iacobellis[177] and Schroeder *et al.*[358] consider the browning reaction as strictly an alkaline sugar decomposition and present evidence that the Maillard reaction is an independent change involving the formation of a glyconyl peptide structure.

Acidity Derived from Sources Other Than Lactose

Minor changes in the acidity of milk upon heating may result from loss of dissolved carbon dioxide, displacement of the calcium phosphate equilibrium and cleavage of phosphate esters of casein. The dissolved carbon dioxide in milk, approximately 4% by volume,[415] contributes 0.01–0.02% acidity.[339] Loss of approximately one half the carbon dioxide may occur during pasteurization, effecting a slight increase in pH. The displacement of the phosphate equilibrium in heated milk causes an acidity increase that tends to compensate the loss of dissolved carbon dioxide. This acidity increase occurs at temperatures above 60°C. as the result of changes in the distribution of soluble and colloidal calcium phosphate. The pH of milk normally decreases with increasing temperature as a result of adjustments in the buffer capacity of the system. The addition of calcium to milk causes the pH to decrease more rapidly, while added phosphate reduces the rate of pH change with increasing temperature.[260] The insolubilization of calcium and phosphorus, as tri-calcium phosphate in heated milk, decreases the pH. A minor formation of colloidal dicalcium phosphate may similarly contribute to heat developed acidity.[86,330] Colloidal calcium phosphates may be recrystallized to hydroxyapatite or decomposed to other more acidic phosphates at temperatures above 60°C.[83,85,86] Cleavage of casein phosphate esters as a further source of acidity in heated milk has been recognized.[290] The phosphates liberated by heat dephosphorylation may further effect a minor acidity increase by tricalcium phosphate formation, through interaction with the calcium salts.[330]

COAGULATION OF CONCENTRATED MILKS

Changes in Milk Induced by Concentration

The stability of the caseinate-phosphate complex to various coagulating agents including heat, rennin, and alcohol, declines rapidly with increasing milk solids concentration. Heat coagulation at a constant temperature tends to increase logarithmically with solids concentration.[157,445] Similarly storage thickening and gelation of heat sterilized concentrates, particularly high temperature–short time processed, are accelerated at higher milk solids concentrations.[10,15,36,137,391] The factors controlling the stability of concentrated milk are even less well understood than those involved in fluid milk stability. The heat stability of evaporated milk bears no apparent relationship to the heat stability of its related raw milk.[158,445]

The pH of fluid milk declines with concentration from an average initial value of 6.6, to approximately 6.2 at 26% total solids.[372] The decrease in pH with increasing milk solids concentration is substantially linear in the 9–40% skim solids range.[170] The pH of nonfat dry milk reconstituted to a 9% solids level is approximately 6.6, while the pH of a 23% solids solution is 6.2 and that of a 40% solution is 6.0.[170] Redilution of concentrated milk with water approximately restores the initial pH. The reversibility of this pH change is presumed to arise primarily from the interconversion of soluble phosphate and tricalcium phosphate. Eilers, *et al.*[78] found that both phosphate and citrate accumulate in the caseinate phase of milk during concentration, the characteristics of the citrate binding by the caseinate phase resembling an adsorption process.

As a result of the high solids concentration developed during freezing or dessication of milk, the pH is further depressed. The unfrozen serum residue separated from frozen concentrated milk by ultrafiltration at $-8°C$. has a pH of 5.8 and a total cation concentration of 0.6–0.8 M.[395] The destabilization of the caseinate phase of frozen milk[369,395] and high moisture milk powder[92,169,200,201] may be related in part to the excessive acidity and high calcium content developed by concentration of the milk solids. For further reference to the coagulation of frozen milk, see Chapter 14.

The activity of calcium and magnesium increases during the condensing of milk, but at a lower rate than that of sodium and potassium, implying formation of insoluble or undissociated salts of calcium and magnesium with increasing milk concentration.[412] Van Kreveld and Van Minnen[411] using an equilibrium exchange resin method, find 6% of the Ca and 16% of the magnesium of milk in the ionic state. These proportions decline to approximately 2 and 5% respectively in sterilized, evaporated milk. The relative activity of the calcium ion decreases more readily than that of the magnesium ion during concentration. However, magnesium ion activity appears to be depressed preferentially during sterilization. Ion activities in sweetened condensed milk are significantly lower than in sterilized evaporated milk, an effect attributed to the lower ionizing power of concentrated sugar solutions.

Nitrogen distribution is not significantly altered by concentration when moderate process temperatures are maintained.[126,259] The rate of serum protein denaturation at 66° and 82°C. is substantially equal for milk solids levels of 9–36%.[126] At intermediate temperatures, a tendency toward lowered denaturation rate with increasing solids is noted.

The relatively low rate of viscosity change with concentration of skimmilk suggests that no appreciable micellar aggregation occurs during concentration of skimmilk. With the attainment of a rheologic concentration of 0.55, the volume per gram of total solids declines to a value of 1.5, from an initial value of 1.9 ml for skimmilk.[78] Changes in the viscosity of skimmilk, that result from concentration, are reversible on redilution of the milk. However, heat sterilization of concentrated milk leads to large viscosity increases that are not fully reversed by redilution, affording evidence of stable micellar aggregate formation. Hostettler and Imhof[162,164] have demonstrated, by electron microscopy, the formation of large, stable, irregular agglomerates in unsweetened condensed milk and drum dried powder. The formation of such aggregates constitutes the basis for the thickening and gelation of condensed milks during sterilization and storage. Micellar aggregation is less evident in sweetened condensed milk and almost absent in spray dried milk. Calculations derived from optical density measurements on skimmilk, indicate that the casein micellar weight averages 180–270 million and increases continuously with concentration.[420] At approximately 50% solids, the average micellar weight in evaporated skimmilk is 280–430 million.

Skimmilk at 45–50% solids, exhibits properties of plastic flow.[112] At this concentration the caseinate micelles are compacted to a degree that renders the system highly vulnerable to coagulation. At the inordinately high solids levels that attain in frozen milk and hydrated milk powder, coagulation of the compacted micelles is impeded by the simultaneous increase of viscosity. The milk solids level at which the relative immobilization of the micellar system dominates the coagulation process has not been determined with any high degree of accuracy. Studies of hydrated milk powders indicate that beyond approximately 88% solids, the rate of casein insolubilization is sharply suppressed.[470]

Effect of Concentration on Heat Coagulation

The coagulation of milk by heat is a function of the milk solids concentration as well as of the temperature and time of heating. The way in which increases in concentration of nonfat milk solids cause the product to coagulate at successively lower temperatures is shown in Fig. 68. The relationship is generally linear with respect to temperature and logarithmic with respect to heating time.[48,157,218,276,445,452,470] Cole and Tarassuk[48] reported finding some deviation from this straight-line relationship when they studied milks heated in the temperature range 110°–160°C. About 12 hr. of heating at 100°C. is necessary to coagulate fresh milk. At 130°C. coagulation occurs in approximately 1 hr. while at 150°C. the reaction occurs in about 3 min.[11,401] While there are wide variations among milks, the time of coagulation of an average evaporated milk (total solids 26%), prepared from milk of good quality, may increase from 10 min. at 131°C. to 60 min. at 114.5°C. and to 7,500 min. at 80°C.

The time of coagulation at a definite temperature varies with the forewarming treatment to which the milk is subjected before concentration. Preliminary heating or forewarming applied to a

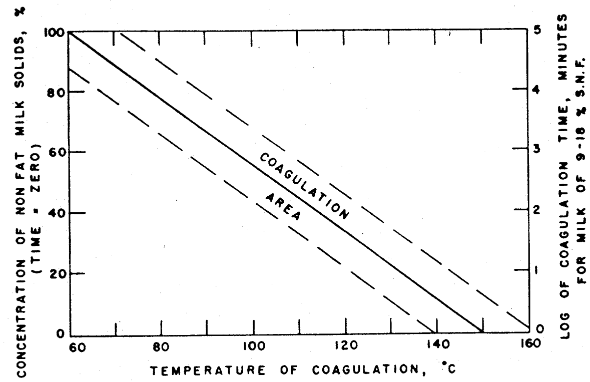
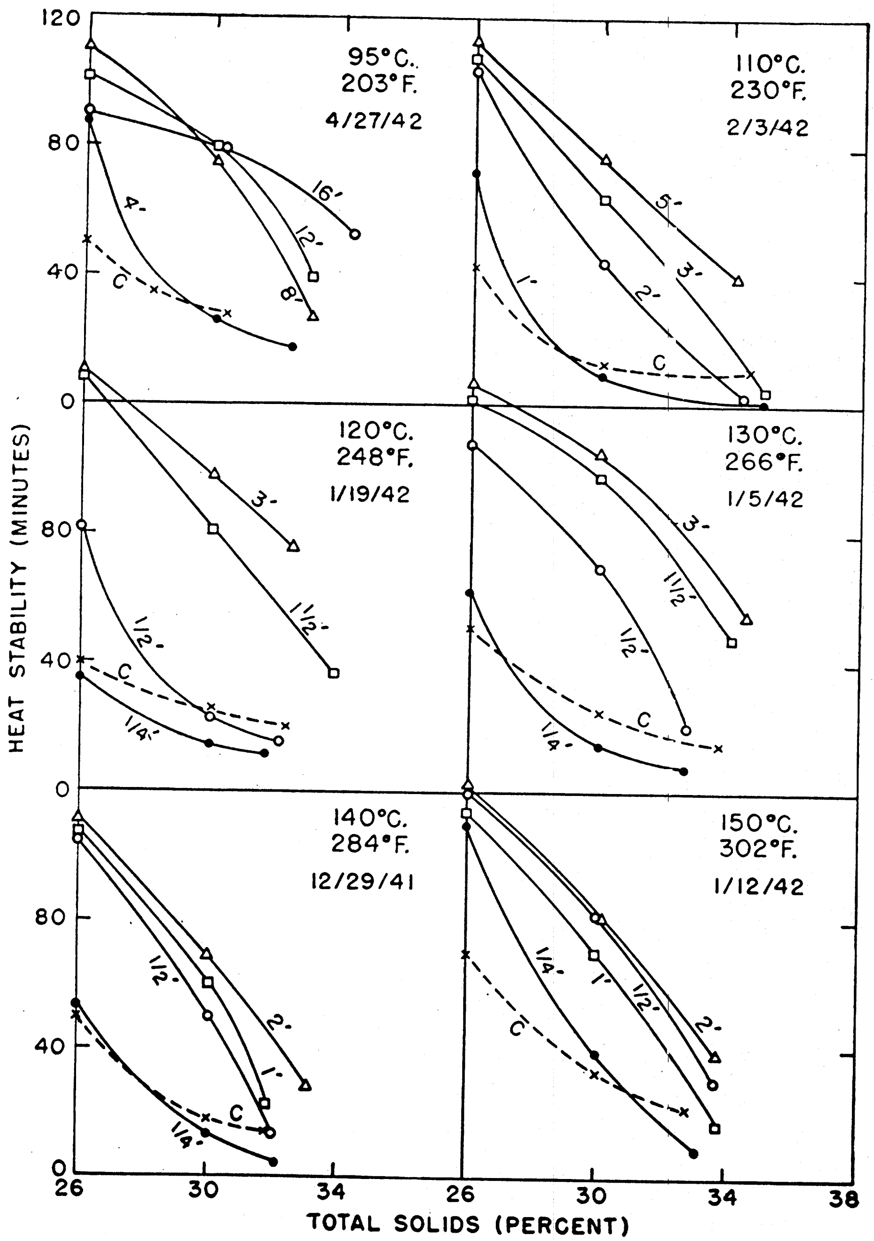


FIG. 68. THE GENERAL RELATIONSHIP BETWEEN THE HEAT COAGULATION AREA OF MILKS, THEIR CONCENTRATIONS, AND THE TIME AND TEMPERATURE OF HEATING

milk not subsequently concentrated, lowers its heat stability. Forewarming continues to cause a lowering in heat stability of milks which are concentrated up to a 13% nonfat-solids level. If a milk is further concentrated to 14% or more solids-not-fat, its heat stability is markedly increased by a proper forewarming treatment.[445] Thus there is a critical solids level, about 13.5%, below which forewarming of the raw milk decreases stability and above which the treatment raises stability. Hence, forewarming is an important manufacturing step in the evaporated-milk industry, where a milk of at least 18% solids-not-fat is to be produced.

No heat-stability test is known which, when used on raw milk, will accurately predict the stability of its concentrated product. It is, therefore, not possible to predetermine the forewarming treatment to apply to a fresh milk so that after concentration it will respond to a sterilization process by producing a required body. The evaporated milk industry determines heat stability and regulates the body of the finished product by making pilot-batch tests and by addition of stabilizing salts. Tests to determine approximate heat stability such as the alcohol,[54] phosphate,[333] or a protein stability test[380,454] are sometimes used on the raw milk. A decision as to the optimum forewarming conditions is usually made on the basis of the behavior of the milk received the previous day.

The quantitative relationship between the temperature-time of forewarming and heat stability, as applied to the long-hold method[217] or to the high-temperature short-time process[16,277,439–441] has been studied by several investigators. Some of these relationships are shown in Fig. 69. Marked variations in stability occur



From Webb et al.[441]

FIG. 69. EFFECT OF VARIATIONS IN THE TIME AND TEMPERATURE OF FOREWARMING UPON THE HEAT STABILITY OF CONCENTRATED MILKS OF DIFFERENT SOLIDS CONTENT

The control sample forewarmed at 95°C. for ten min. is marked "C." All other samples were forewarmed by the HTST method in a Mallory heater and held for the number of minutes indicated for each curve. The forewarming temperature for each series is indicated. Milk for each sub-figure was obtained on the date shown. Heat stability refers to the time necessary to initiate coagulation in cans at 115°.

with variations in forewarming temperature and time. Rapid improvement in resistance of the concentrate to heat coagulation results with increasing forewarming temperature. Milk to be concentrated and sterilized as evaporated milk is usually forewarmed in a hot well by direct steam injection to raise it to 95°C., where it is held for 10–20 min. The stability thus imparted to the milk will usually enable it to withstand heat sterilization as a 26% solids evaporated milk. Greater heat stability can be obtained by the use of high-temperature forewarming carried out by injecting steam directly into the milk or by forcing the milk through a pressure-heating system, usually in the form of a tubular heater. As the temperature of forewarming is raised, shorter holding times are necessary to attain maximum heat stability. For the manufacture of a 3-to-1 sterilized milk of 35% solids content, a high-temperature forewarming treatment within the range of 115°C. for 2 min. to 138°C. for 15 sec. will usually be required. The forewarming of sweetened condensed milk, discussed later, must be conducted differently from that of evaporated milk.

The rapid decrease in the heat stability of milk with increasing concentrations makes it impractical to attempt to sterilize a product which has been concentrated to a ratio substantially higher than 3:1 (27% nonfat solids). In commercial practice evaporated milk concentrated 2:1 can be sterilized under several temperature-time combinations such as: 115°C. for 16 min., 130°C. for 2 min., and 140°C. for 3 sec. Milks concentrated to 3:1 can sometimes be adjusted by forewarming and salt-balance procedures so that they will withstand the heat necessary for sterilization. The daily processing of such a concentrate by the usual sequence of processing steps would be difficult, and with some milk supplies, almost impossible. The relatively low heat stability of concentrated milks may be avoided in high temperature sterilization processes by sterilizing the milk prior to aseptic homogenization and canning.[79,378]

The Viscosity of Sterilized Milk Concentrates

Viscosity control during processing and storage is an important consideration in dairy-product manufacture. Pre-coagulation thickening occurs in the manufacture and storage of evaporated, plain and sweetened condensed milks, ice cream mix, and various specialty products. Thickening of milk precedes its coagulation by heat, and increases rapidly with increasing concentration. The actual process of thickening occurs in a relatively short period of time, just prior to separation of the curd. Coagulation is easily determined by visual appearance of curd aggregates but thickening is a less perceptible change. Since thickening is a transient state, it is often difficult to stabilize a milk concentrate at any desired viscosity. Evaporated milk must be treated so that the sterilization heat brings it almost to, but never beyond the point of coagulation. Here the thickening reaction produces a desirable creamy body although some thinning occurs during storage. The rate of thickening during sterilization in the can at 116°C., although variable, becomes greatest in the ten minutes preceding coagulation.[59] Milks of high heat stability which reach the end of the sterilization period before entering the thickening phase do not develop the high viscosity shown by milks of lower heat stability.

Evaporated milk is subjected to conditions of agitation that vary considerably in different sterilization processes. A heavy-bodied milk will not form under the severe agitation sometimes used to attain rapid heat exchange. However, Gammack and Weckel[100] using an intermittent hold, Ball[10] by the addition of lactic acid to raw milk, and Tarassuk *et al.*[389,390] by a proteolytic enzyme treatment before concentration, produced evaporated milks which (after sterilization) had higher viscosities than untreated control samples.

In the case of sweetened condensed milk, excessive thickening developed during manufacture will lead to an objectionable gelation in storage. Thickening may be caused by improper forewarming, high acidity in the milk, high concentration of solids, high storage temperature, and other factors.

Sweetened condensed milk should be forewarmed at a lower temperature than evaporated milk to avoid storage thickening. The effect of time and temperature of forewarming of milk on the viscosity of its sweetened condensed product has been studied by several investigators.[217,310,375,447] Representative values are shown in Table 93. Although forewarming at 71°C. produces a low

TABLE 93

EFFECT OF TIME AND TEMPERATURE OF FOREWARMING ON THE VISCOSITY OF SWEETENED CONDENSED SKIMMILK[a]

Forewarming Conditions		Viscosity after Storage at 15.5°C. in Poises		
°C.	Min.	1 Day	24 Days	58 Days
71	10	25	40	95
82	10	360	534	853
95	10	570	846	...
115.5	0.5	20	30	69

[a] The data are those of Webb and Hufnagel[447] except that the values at 71°C. for 10 min. were estimated from references cited in the text.

viscosity product, this temperature does not effectively destroy many bacteria, yeasts, molds, and enzymes, the presence of which often produce body and flavor defects during storage. It is commercial practice to forewarm the milk at about 82°C. This produces a product of sufficient viscosity to retard separation of fat and crystallized lactose during storage, yet one which will not gel when held at room temperature or lower for several weeks. When high-temperature short-time heating equipment is available, forewarming at 115°C. for less than a minute may be used to produce low-viscosity sweetened condensed milk for shipment to warm climates. Thickening is usually measured and expressed as relative viscosity and an attempt generally is not made to determine plasticity. Typical relative viscosities of some dairy products are shown in Table 94.

TABLE 94

RELATIVE VALUES FOR THE PRE-COAGULATION VISCOSITIES OF SOME DAIRY PRODUCTS

	Relative Viscosity after Storage at 21°C. (in Centipoise)		
	No Storage	1 Month	1 Year
Evaporated milk			
Sterilized in the can			
1. Batch sterilizer[a]	80	63	45
2. Continuous sterilizer[b]	24	15	14 (6 mo.)
Sterilized by HTST	15	15	17 to gel[e]
Sweetened condensed milk[c]	6,900	9,200	36,800
Concentrated skimmilk[d]			
a. No heat treatment after concentration	200	Perishable	...
b. Heated without agitation	55,000	Perishable	...
c. Same as (b) but homogenized after heating	2,500	Perishable	...
d. Same as (b) but severe agitation during heating	1,800	Perishable	...
Ice cream mix	64	Perishable	...

Source of information:

[a] Webb, Deysher, and Potter.[442]
[b] Tamsma and Tarassuk.[389] Storage at 25°C.
[c] Webb and Hufnagel.[449]
[d] Webb and Hufnagel.[448] Milks of 36% solids heated under various conditions of agitation to first appearance of coagulation.
[e] HTST processing may result in wide variations in final viscosity; the less heat used the more viscous the aged product. Based on authors' unpublished data.

Completion of the sterilizing process does not mark the end of the effects of time and temperature on the stability of the concentrated milk system. Changes continue but at a rate dependent not only on previous heat treatments but also on time and temperature of storage. Most products thicken with age but evaporated milk nade by the usual longhold sterilization method thins to a basic 'iscosity during the first few days of storage.[59] In contrast, high-emperature short-time (HTST) sterilized milk (135°C. for 30 sec.) hickens until it gels in storage. The rate of thickening decreases as he processing heat increases.[15] The viscosity of sweetened ondensed milk increases logarithmically with the temperature of torage and arithmetically with storage time.[449] For conditions of onstant viscosity, time varies logarithmically with storage temper-ture. Whether the gelation of HTST sterilized milks in storage epresents an extension, at a slow rate, of the heat coagulation eaction, is uncertain.[391] Thickening during the storage of HTST oncentrated milks proceeds rapidly after an initial increase in iscosity. The more severe the heat treatment to which samples re subjected the more stable are the milks against thickening. amples of 2:1 milks processed in a tubular heater at 135°C. for) sec. and subsequently heated in cans at 115°C. for 2, 6, 12, and 3 min., start to thicken (20 cp.) at 32, 36, 41, and 51 weeks respec-vely.[15]

Many workers[10,36,137,391] have shown in various ways that the onset gelation is hastened by lessening exposure of the milk to heat uring the sterilization process, as in HTST processing. Milks erilized by irradiation without heat, gel quickly in storage.[151–153] his gel forming property of irradiated milk is probably related to ie same mechanism that causes gel formation in milks processed ith a minimum of heat during sterilization. There is no correlation etween long continuing fluidity and high heat stability in evaporated ilk.[15] Heat appears to more effectively stabilize milk against elation in storage when it is applied to milks of high solids content 6–34%), than when used on milk of normal concentration.[391] eat applied to milk concentrates at a solids level of 32–45%, llowed by dilution to 26%, imparts to the diluted milk a greater sistance toward age-thickening.[378] Thickening is retarded by low mperature storage.[100,365]

There has been intensive effort to devise a sterilization process for eparation of a beverage quality concentrated milk at the 2:1 or 1 level by manipulation of time and temperature of heating. The jective has been the production of a concentrate with a minimum cooked flavor, discoloration, fat separation, thickening, and aling in flavor during storage.[16,27,36,79,275,309,378,444,459,460] Of these fects thickening and gelation have been the most obvious and jectionable. An acceptable sterile concentrate can be prepared by e use of optimum processing conditions followed by storage at

10°–15°C. A typical process sequence would include forewarming at 115°C. for 2 min., concentration not to exceed 3:1, sterilization at 135°C. for 30 sec., cooling to at least 98°C., aseptic homogenization at 4000 p.s.i., cooling to 20°C., and aseptic canning. Passing the uncooled canned milk through a hot water bath at 72°C. for 10 min., further delays gelation but increases cooked flavor. Both flavor and physical stability are favored by refrigerated storage. The proposal has also been made that raw milk be sterilized, then aseptically homogenized, concentrated, and packaged.[27] This procedure avoids coagulation problems during processing but does not delay storage thickening.

Sediment may form in HTST sterilized concentrated milks when their heat stability has been exceeded and the coagulum is subsequently redispersed by homogenization or by excessive agitation during turbulent flow in heating or cooling tubes.[462,465] Much experimental work has been done recently on various aspects of sediment formation but the problem is still one that requires intensive research.[73,166,359,462–464,466,467]

The protein of the sediment of stored evaporated milk exhibits a different electrophoretic pattern than that of nonsedimented protein.[166] Homogenization promotes the development of fat-protein complexes[94] which may contribute to storage sedimentation.

One process for retarding gel formation adjusts the calcium-sodium ion ratio of the milk by use of an ion exchange resin.[377] Calbert and co-workers[36,103] have sought to stabilize HTST processed milk by holding it after sterilization at 94°C. to permit a critical viscosity development. The incipient coagulum is next destroyed by homogenization and it is then not expected to reform during storage. One problem in the use of this procedure is to so adjust the degree of thickening that, when the product is finally smoothed out by homogenization, neither sediment formation nor gelation will occur in storage.

Polyphosphates effectively retard age thickening and gelation in HTST sterilized milk concentrates.[221] Early work indicated these salts had a rather uncertain stabilizing effect[64,428] but optimum quantities of polyphosphate glass having an average of 4.8 phosphorus atoms per chain now appear to afford effective stabilization.[221] In unpublished work, Leviton[219] places an optimum concentration, for a sterilized concentrate, at 0.6 lb. polyphosphate per 100 lb. milk solids, thereby extending storage life of a 3:1 concentrate at 21°C. from 50 to 347 days. The kind of phosphate salt used as a stabilizer is important since the common Na_2HPO_4 and some other phosphates increase gel formation during storage.[74,219]

The storage gelation of HTST evaporated milk does not appear to be caused by bacterial action.[167,410] There is still some question whether enzyme action is involved. Research on the phosphatase test in milk indicates that this enzyme is destroyed during pasteurization and could not be expected to survive an HTST treatment.[140,216,355] However, it has been demonstrated that certain phosphatases in HTST treated milk products may reactivate after apparent heat destruction.[97,365,471–473] Thus it is conceivable that proteases present in milk, and considered destroyed by HTST treatment, could be reactivated during storage and cause gelation. Clarification of the mechanism of storage gelation in HTST sterilized milks must await further research.

Salt Balance and Heat Stability of Concentrated Milks

Sommer and Hart[370–373] first showed that a critical balance between the natural acidic and basic salt components of milk appeared necessary to provide maximum stability to heat coagulation. In most cases inadequate resistance to coagulation related to the presence of an excess of calcium and magnesium. The addition of phosphates, citrates, or carbonates to such milks was shown to improve their heat stability appreciably. It is generally assumed that the salt balance directly affects the heat stability of the casein but this balance may operate indirectly on the casein through its effect on denaturation and interaction with the serum proteins.[277] The relation between the mineral composition of milk and its heat stability has been studied intensively, but the distribution and role of the milk serum electrolytes at high temperatures remains obscure.[82,330,348,452]

When milk is preheated and concentrated, the effect of acid and stabilizing salts may be the same or opposite to that observed in the original raw milk. Evaporated milk may respond to small changes in pH in the same manner as fluid milk but its pH sensitivity in the presence of added β-lactoglobulin is much less than that of fluid milk.[347] Slight natural variations of the raw milk between pH 6.58–6.65 do not affect the heat stability of the concentrated product.[18]

Three types of concentrated milks are shown in the lo..er curves of Fig. 70. One kind of milk is stabilized by addition of ortho-phosphate or citrate, another is stabilized by addition of calcium or magnesium, and a third is destabilized by salt additions.[158,445]

The three types of milk are usually derived from a raw milk, of which the curve shown in upper Fig. 70 is typical.

Concentrated milk III, stabilized by small quantities of $CaCl_2$ or other chloride including hydrochloric acid is comparatively rare,

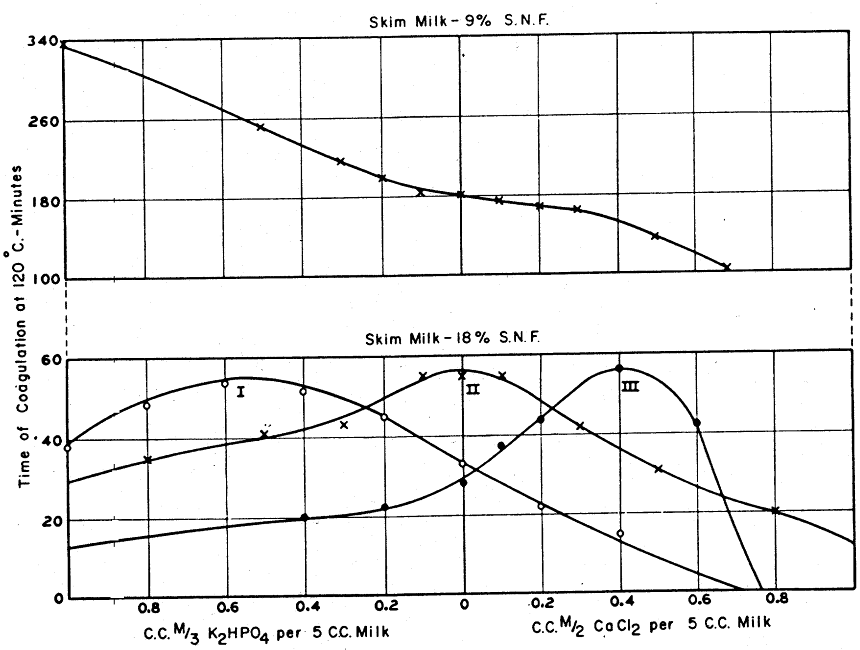
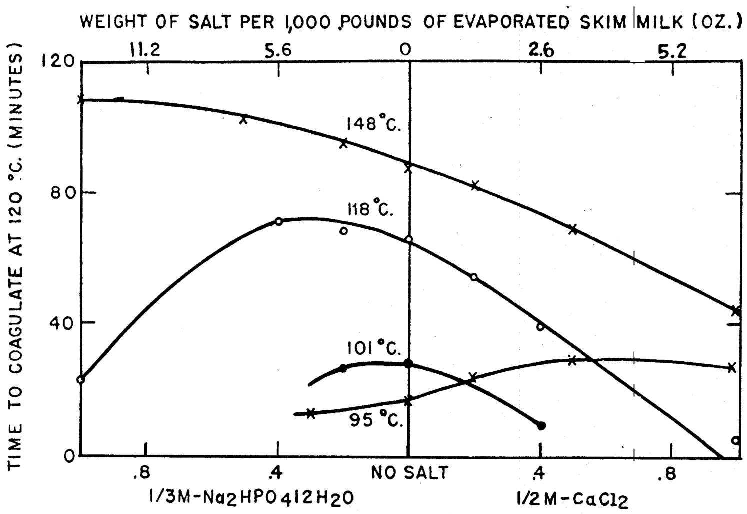


FIG. 70. THE EFFECT OF THE ADDITION OF VARYING AMOUNTS OF DIFFERENT ELECTROLYTES UPON THE HEAT STABILITY OF NORMAL AND EVAPORATED SAMPLES OF THE THREE DIFFERENT TYPES OF MILK

apparently being secreted by from 10 to 20% of the cows of a normal herd. If a Type III raw milk is placed in storage, there will be a gradual shift in the heat stability curve of its evaporated product through Type II to Type I, although little change may be noted in the stability curve of the unevaporated sample. The shift from Type III to Type I will be accelerated by the development of lactic acid during storage,[438] but it may also proceed without the accompaniment of a measurable change in pH.

There is no clear division among the three types of milk, since they merge into one another and numerous curves can be obtained showing a difference in degree of variation. There may be a shift from type to type as a result of a change in forewarming temperature as shown in Fig. 71.[439] Thus a milk forewarmed at 95°C. was heat stabilized by calcium after concentration. When higher fore-



From Webb and Bell[439].

FIG. 71. THE EFFECT OF STABILIZING SALTS AND FOREWARMING TREATMENTS UPON THE HEAT STABILITY OF EVAPORATED SKIMMILKS

warming temperatures were used, both calcium and phosphate destabilized the milk but some stability was obtained at certain critical levels of phosphate.

Many different salts may be used with substantially the same results as are obtained with $CaCl_2$ and Na_2HPO_4. Differences in the valency of the ions concerned generally account for the differences in ionic concentration found necessary to produce a given result. The chlorides of H, Na, K, Ca, Mg, Ba, Al, and Th furnish a source of strong cations while the sodium and potassium citrates and orthophosphates may be used when strong anions are required. In general, calcium has a greater impact than phosphate on heat stability. The addition of small quantities of phosphate to a milk to increase heat stability is useful if a phosphate stabilized milk is encountered (curve I, Fig. 70). However, if the milk is stabilized by calcium (curve III), the addition of phosphate does not increase stability, but at the same time it effects no marked lowering of stability. This fact accounts for the success of phosphate salts in evaporated milk manufacture.

The addition of salts to milk before forewarming and condensing modifies the heat stability curve of evaporated milk. If a small quantity of calcium chloride is added to a fresh milk which normally has a stability curve of a type of milk II, Fig. 70, the resulting curve is shifted toward curve I. If a phosphate salt is added to the fresh milk, the heat stability curve is shifted toward curve III. This behavior is to be expected since it is the normal adjustment arising from an excess of either a strongly positive, or negative ion, for this particular milk.

From the foregoing discussion it can be seen that the effect of salts on the heat stability of milk is largely empirical but of paramount importance in the manufacture of evaporated milk. The stabilizing salts that have been approved by the Food and Drug Administration[90] are calcium chloride, sodium citrate, and disodium phosphate. The hearing record shows that these salts are needed in evaporated milk manufacture to control heat coagulation in certain types of milk and at certain seasons of the year. Their use is permitted to the extent of 0.1% of the weight of the evaporated product.

An exception to the stabilizing influence of ortho-phosphate has been noted. Stewart[378] advocates use of forewarming rather than phosphates to obtain stabilization in HTST sterilization. The principal defect in this product, gel formation in storage, can be minimized if phosphate addition is avoided in processing.[219,378]

While the addition of salts to milk is a simple method of changing its heat stability, this can also be done by removing ions from the system. Skimmilk treated to remove 60% of its calcium may be added in an amount equal to 0.5–2.5% of the batch of original milk, and the mixture processed into evaporated milk.[184,292,293] Stabilization by this means is equal to the use of from 2 to 7.3 ozs. of disodium phosphate. Evaporated milk of 40% solids can be stabilized against heat coagulation by the use of a mineral-ion exchanged milk as a ten per cent replacement for normal solids.[193]

Effect of Nonionic Substances

Nonionic and inert materials such as fats, starches, sugars, and vegetable pulps usually promote protein coagulation during heating of milk systems in which they are dispersed. Suspended particles appear to adsorb protein, finer dispersions tending to concentrate the protein with increasing effectiveness. This accumulation of protein on the interphase tends to lower protein stability by encouraging local coagulation which quickly destabilizes the entire system. Ground casein and vegetable pulps, decolorizing carbon, and ground filter paper are among the substances that have been shown to lower heat stability.[457] Sugars including lactose, sucrose, and dextrose usually promote heat coagulation although dextrose under certain conditions exerts a stabilizing influence.[457]

The destabilizing effect of sugars and starches on the heat stability of milk protein systems is significant in the manufacture of certain foods processed at high temperatures.[446] Sterilized cream-style soups, sauces, and creamed vegetables must have a smooth body free from lumps and visible curd. Wheat flour, corn or potato starch are common thickeners which promote coagulation of the protein. Since coagulation in most products of this type cannot be avoided the material must be handled so that a soft, smooth, gel type of coagulum is produced. Milkfat in its normally dispersed state affects the heat stability of the system but slightly.[58,157] Homogenization, however, increases the dispersion of the fat and creates new surfaces for adsorption, simultaneously concentrating the protein on these surfaces, forming a fat-protein complex.[94] Clumping of the newly formed globules often occurs and this further concentrates and destabilizes the protein. It follows, therefore, that those factors which influence the degree of fat clumping will also modify the heat stability of the product. The effect of homogenization on heat stability is slight at low fat levels[38,155,404] but becomes appreciable with increasing fat content.[175]

The specific conditions of homogenization have an important bearing on the heat stability of fluid milk products. Preheating temperature, homogenization pressure, fat and solids-not-fat concentrations, and salt equilibria, were shown years ago to affect the fat clumping and coagulability of milks and creams subjected to homogenization.[61,63,249,405,437,443] The general nature of these relationships are shown in Fig. 72. Rehomogenization, or the use of a second stage valve, wherein the second pressure is lower than the first, breaks up the larger clumps and consequently increases heat stability over that observed after a single homogenization.[62,249,437] Increasing homogenization temperatures favor heat stability of concentrated milks when the homogenization is practiced before forewarming and condensing.[246]

The feathering of homogenized creams when added to coffee is a form of heat coagulation. Coffee cream is usually homogenized to retard fat separation and to impart a smooth creamy body. The effect of homogenization on the heat stability of 20 and 30% cream is shown in Fig. 72. The salt content of the cream or of the coffee, chiefly the presence of relatively high levels of calcium in either, is an important factor.[405] Acid development before or after processing quickly renders cream susceptible to feathering. King[196] lowered

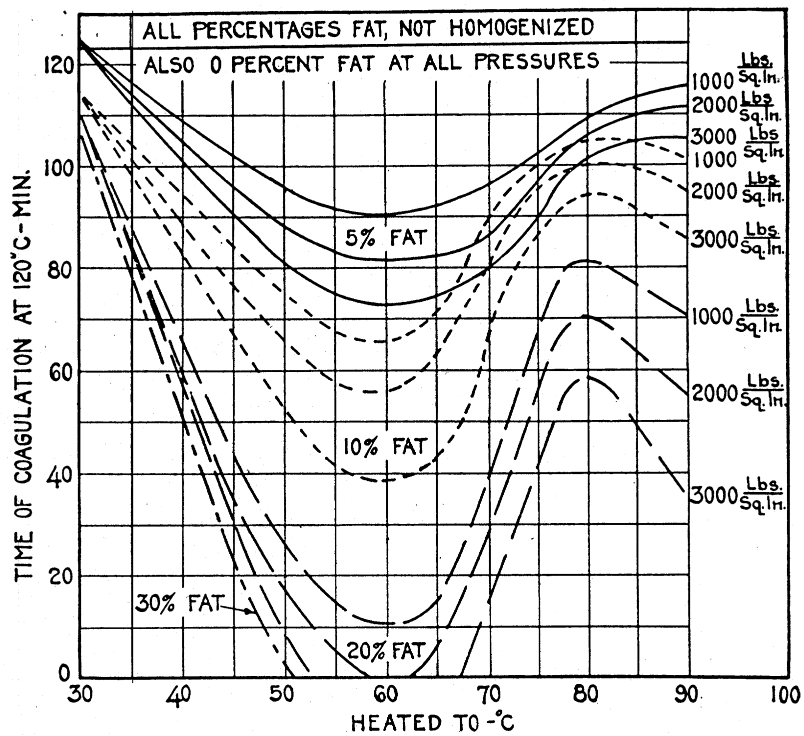


FIG. 72. VARIATIONS OF COAGULATION TIME OF MILK OF NORMAL SOLIDS-NOT-FAT CONTENT WITH CHANGES IN FOREWARMING TEMPERATURE, FAT CONTENT, AND HOMOGENIZATION PRESSURES

No homogenization below 50°.

the feathering value of cream by adding to it five per cent of skimmilk treated with an ion exchange resin to lower its calcium content.

In the processing of milk, coffee cream, and evaporated milk, the destabilizing effect of homogenization is incidental to the primary objective of retarding fat separation in storage. The interrelationships of viscosity, heat stability, conditions of homogenization, and storage, on fat separation in evaporated milk have been studied.[220,247–249,442] Trout has reviewed work on the homogenization of milk from all aspects.[407]

Coagulation of Milk by Alcohol

Interest in alcohol (ethanol) coagulation of milk has continued in part because of the possibility that it might furnish evidence for the stability of milks toward heat. However, experience has shown that alcohol coagulation is not a sufficiently reliable measure of the susceptibility of milk to heat coagulation. The alcohol test is usually made on samples of raw milk by rapid addition of about 2 cc. of 68 or 70% alcohol to an equal quantity of milk in a test tube.[54,139] Coagulation of the casein on shaking indicates a positive test. In general, milks which are not precipitated by 70% alcohol tend to be sufficiently stable to withstand sterilization in evaporated milk manufacture, though occasionally milks stable to heat will give a positive reaction.

As milk gradually sours it becomes susceptible to alcohol precipitation shortly before it loses its stability to sterilizing temperatures, though the precise pH at which the change takes place is not the same in all milks. As instability thus induced is constantly encountered in the industry, the usefulness of the test is obvious. Milks are not infrequently encountered, however, which though otherwise of good quality yield a positive alcohol test reponse when secreted. Colostral milk is always alcohol positive. Milk secreted at the end of lactation, or when the mammary tissue is slightly irritated or inflamed, though suitable for food is usually unstable to heat and, as a rule, is alcohol positive.

The alcohol test was originally suggested as a measure of the freshness and purity of market milk, but its unsuitability for this purpose was shown by Ayers.[7] Susceptibility to coagulation by alcohol has been found to vary with milks drawn from different quarters of the udder of the same cow,[17] as a function of the feed,[70] and of the time and temperature of holding after milking.[342]

The extent to which the different milk constituents affect coagulation by alcohol is obscure. Addition of small quantities of calcium or magnesium induce positive test results[370] but it is difficult to correlate the heat stability of an evaporated milk with alcohol tests made on the original milk.[445] No correlation was found between alcohol coagulation and stability of sweetened condensed milk against age thickening.[376] White and Davies[451,452] concluded from a detailed chemical examination of many milk samples, that the strength of ethanol required to coagulate the caseinate complex was inversely related to the concentration of ionized calcium in the milk.

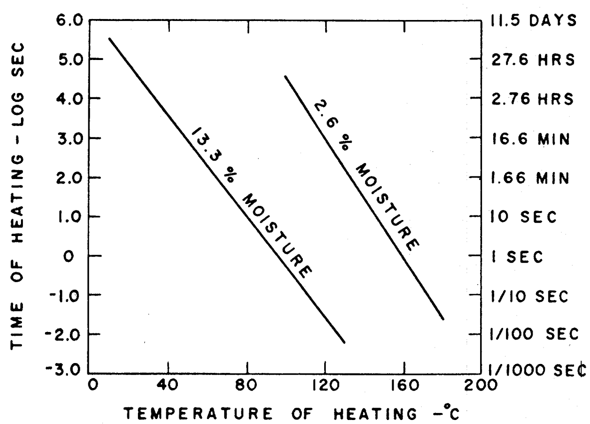
Solubility of Dried Milks

Effect of Heat.—The native properties of the milk components are substantially unmodified by moderate milk drying conditions. In freeze dried milk, the equilibrium ratio of α and β lactose and the salt distribution remain essentially intact. The normal size dispersion of the caseinate phase and its clottability by rennin are substantially recovered on reconstitution of the dried product. Depending on the preheat conditions, drier design and temperature of operation, the properties of spray dried powder may vary significantly. The initial temperature of an evaporating milk droplet, in a spray drier with parallel air-flow, does not appreciably exceed the unit bulk temperature, and can be effectively held to temperatures below 60°C.[49,197,241] As the falling rate period is approached, in the course of further evaporation, the temperature rises to a final value determined by the final temperature of the drying gas and the residence time in the drier. Under properly controlled spray drying conditions, the changes in milk protein structure and solubility are minor. Spray drying does not denature the serum proteins significantly, and the level of serum protein denaturation in the dry milk is substantially equal to that of the condensed milk from which it is processed.[126]

Loss of solubility of dry milk during processing or storage is largely a manifestation of changes in the stability of the caseinate-phosphate complex. The solubility of spray dried powder is unaffected by preheat treatments of the fluid milk that cause extensive serum protein denaturation.[53,240] Exposure of dry milk to a dry heat treatment sufficient to cause total insolubilization of the caseinate phase, may have negligible effect on the serum protein. In a maximally insolubilized nonfat dry milk, the total casein comprising 35% of the solids, may be rendered insoluble, while the solubility of the serum constituents remains intact. Jenness and Coulter[181] suggest that preheat may exert a stabilizing influence against coagulation during drying, similar to the benefit of preheat in evaporated milk sterilization.

While preheat conditions do not significantly affect the initial solubility of dried whole milk, powders processed from high preheat milk tend to develop insolubility, during subsequent storage at 37°C., more rapidly than low preheat powders. The free fat in dry whole milk decreases somewhat on storage and appears to be related to solubility decrease and film formation in the reconstituted milk.[229]

Under normal spray drying conditions the casein solubility is spared. At excessively high exit air temperatures, the casein in spray dried milk powders may insolubilize at a rate that is approximately logarithmic with the temperature.[402] The conditions that prevail during roller drying of milk alter the solubility of the casein extensively. The film of milk on the heated drum has a residence time of several seconds, during which it attains progressively increasing solids concentrations upon continuous evaporation. At solids levels above 60%, the casein is particularly vulnerable to the high temperature of the drum surface. Coagulation is essentially instantaneous at solids concentrations exceeding 80%. Wright[470] determined that each increase of one per cent in milk solids lowered, by 1 °C., the temperature required to effect a constant degree of insolubilization for a fixed heating period. The maximum quantity of protein rendered insoluble by any moist heat treatment approximates 75% of the total. At a fixed moisture level, the time required to produce a constant degree of insolubility is a logarithmic function of the temperature. This relationship is shown in Fig. 73. The rate



From data by N. C. Wright[470]

FIG. 73. THE TIME-TEMPERATURE RELATIONSHIP FOR THE DEVELOPMENT OF 50% PROTEIN INSOLUBILITY IN NONFAT MILK POWDER HEATED AT 2.6 AND 13.3% MOISTURE

Solubility measured at 20° C.

of protein insolubilization increases nearly fivefold for each 10° rise in temperature. The slope of this curve is similar to that derived from measurements on the heat stability of evaporated milk. Similarly, the coagulation time for milk powder at 86.7% solids is extrapolable from the heat stability curves of concentrated milks of 28–38% solids levels, suggesting that the heat coagulation of evaporated milk and insolubility of dried milk may have a common origin. At moisture levels below 13%, the rate of insolubilization of heated milk is sharply reduced. Nevertheless, a considerable solubility loss is sustained by milk powder with a residual moisture below three per cent, when subjected to a dry heat treatment (see Fig. 73).

The "solubility value" of the protein in milk powder is a function of the temperature and energy applied to effect its redispersion in water to colloidal dimensions.[168] The solubility of dry heated powder varies with the temperature of reconstitution, whereas the protein in high moisture powders is irreversibly insolubilized.[169,470] With increasing temperature of reconstitution, the full solubility of the protein in dry heated milk can be recovered. The solubility of milk powder in 20° and 50°C. water is therefore a useful criterion for differentiating dry heat from moist heat insolubility. Howat and Wright[168,169] demonstrated that protein insolubility in roller dried milk is partially reversed at elevated temperatures of reconstitution. The momentary final contact of the dry milk film with the hot surface of the drum during roller drying, therefore, accounts for a significant proportion of the total insolubility of roller dried milk powder. Wright[470] considers dry heat insolubility a physical, rather than a chemical, change in the casein since the rate of insolubilization is directly proportional to the time of heating, and is independent of the quantity of unchanged casein. The casein in milk powder treated with absolute alcohol undergoes a similar insolubilization, that is also reversible at higher temperatures of reconstitution. A dehydration of critical water, such as loss of water of imbibition, is therefore suggested as a possible cause of dry heat insolubility.

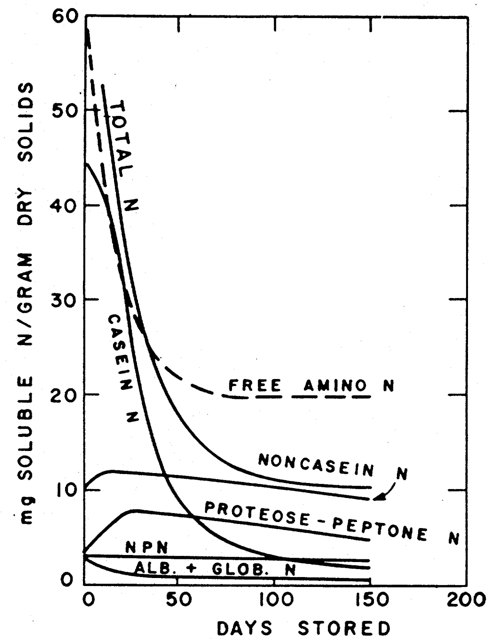
The solubility changes in dry heated milk powder are wholly related to destabilization of the caseinate complex, with no detectable alteration in the serum protein and nonprotein nitrogen constituents.[470] The insolubles of dry milk, manufactured by different processes, are generally of similar composition and comprise casein together with calcium and phosphorus in a ratio suggesting tricalcium phosphate.[423] In roller dried whole milk, some fat appears to be associated with the insoluble caseinate complex.

Effect of Storage Conditions on Stability of Dried Milks.—The stability of dry milk during storage is critically affected by the moisture content and storage temperature. High moisture levels, due to inadequate dehydration or reabsorption of atmospheric moisture, promote insolubilization at relatively mild storage temperatures. The rate of solubility loss is a function of both moisture concentration, and temperature.[50,51,135,136,200,201,204,385] Below five percent moisture, solubility changes are relatively insignificant at normal storage temperatures. In a study of the solubility changes in hydrated nonfat dry milk, Henry *et al.*[135,136] found that the temperature coefficient of the reaction leading to insolubility exceeds a value of five at temperatures above 20°C. Consequently, the solubility of moist powders may remain unchanged for long storage periods at 20°C., but fails rapidly at 37°C. The solubility of nonfat dry milk, hydrated to 7% moisture, fails in 400, 80, and 10 days when held at 20°, 28.5°, and 37°C. respectively, and when reconstituted in 20°C. water. Initially, the insolubility is reversible on reconstitution in 50°C. water, resembling the differential solubility of dry heated milk powder or roller dried powder. The initial solubility of nonfat dry milk can be retained for 700 days at 37°C. storage if the moisture level does not exceed 4.7%.

The occurrence of solubility loss in powder of 7.6% moisture, is coincident to crystallization of the lactose, as a result of which the equilibrium relative humidity increases from 42 to 55%. The change in activity of the water due to lactose crystallization may contribute to the overall reactions leading to solubility loss. The change in stability is nonbacterial and the rate appears to be unrelated to the gas atmosphere in the container. The insoluble component is predominantly casein which can be totally insolubilized during prolonged storage at 37°C., in powder hydrated to 7.6% moisture. Solubility of the lactalbumin and lactoglobulin components is similarly impaired. Changes in the distribution of soluble nitrogen in hydrated milk powder are shown in Fig. 74.[136]

A number of significant changes occur in high moisture milk powder, concurrent with the loss of solubility. The lactose is gradually bound by the protein, attended by a parallel reduction of free amino nitrogen. The pH decreases steadily, and the characteristic changes associated with the Maillard reaction between sugars and amino nitrogen become evident, including development of brown discoloration and production of carbon dioxide, reducing substances, and fluorescing compounds. Stale and caramelized flavors also develop rapidly in milk powder under conditions of high humidity and elevated storage temperatures and a significant loss in biological value of the protein is incurred. Changes in nonfat dry milk hydrated to 7.6% moisture and aged 100 days at 37°C. include a nearly total loss of protein solubility, a pH decrease of 0.4 of a unit in the reconstituted milk, crystallization of 80% of the lactose, destruction of 70% of the original amino nitrogen, and approximately 6% of the lactose.[136]

Most of the deteriorative changes in moist milk powder are attributed to a 1:1 interaction between the free amino groups of the milk proteins, largely the ϵ-amino groups of the lysine residues, and the potential aldehyde group of lactose.[136] The initial complex is solu-



Data from Henry et al.[136]

FIG. 74. CHANGES IN THE DISTRIBUTION OF THE SOLUBLE NITROGEN IN NONFAT MILK POWDER CONTAINING 7.3% MOISTURE, STORED AT 37° C.

Simultaneous changes in free amino nitrogen, determined by the Van Slyke method and expressed as mgm per gm of protein nitrogen, are shown in the dashed curve.

ble and colorless and as much as 65–75% of the reactive amino nitrogen may be bound before appreciable solubility or color change becomes evident.[136,212] The reaction rate is largely determined by the activity of the moisture. The temperature coefficient exceeds six for milk powders of 7.6% moisture, decreasing to a value of two at moisture levels below 5%. The progressive loss of amino nitrogen during storage is paralleled by a decrease in lactose and an increase in the weight of the nondialyzable fraction. The complexed sugar becomes irreversibly bound and, through a series of undefined degradative changes, yields the brown colored pigments characteristic of the Maillard reaction. The reaction in moist powder consumes a maximum of 70% of the total free amino nitrogen as determined by Van Slyke analysis.

While the loss of protein solubility in dry milk is generally attributed to the sugar-protein interaction, the mechanism is obscure.

Henry *et al.*[136] speculate that insolubility may finally arise from an induced denaturation of the protein molecule as a consequence of its complex formation with lactose, or subsequent degradation reactions. This process bears analogy to the storage insolubilization of dried egg white.[12,198,244,379]

On the basis of amino nitrogen binding capacity, the relative order of reactivity of the various sugars toward casein is xylose > arabinose > glucose > maltose > lactose > fructose.[222] Lea *et al.*[121,211,213,215] established that the ϵ-amino group of lysine is the primary reaction site in the casein-glucose system. The decrease in amino nitrogen is at a maximum in the 65–70% relative humidity range, and the temperature coefficient, at 15°–25°C., is 5.4. The greater reactivity of glucose induces more rapid insolubilization of the caseinate complex in addition to intensive discoloration. In freeze dried preparations of various sugar-milk protein mixtures, stored at 55% R.H., sucrose is unreactive, while glucose inactivates 70–80% of the available amino groups, although at a concentration of only $^1/_6$ equivalents.[212]

The interaction of lactose and casein is a contributing factor, but is not essential, to the development of insolubility in high moisture milk powder. The already high acidity in concentrated milk solids is further increased as a consequence of sugar protein interaction and this secondary change may have an additional important influence on solubility. Insufficient attention has been directed toward the observation that the caseinate complex, in the total absence of sugar, will insolubilize at 55% R.H.[102,130] While glucose appears to accelerate the development of insolubility, casein in the absence of sugar may insolubilize more rapidly than casein in the presence of lactose. Furthermore, sucrose protects the solubility of the casein, and the insolubilizing effect of glucose, in the presence of sucrose or lactose, is largely suppressed. It is interesting to note that centrifugally compacted caseinate micelles are initially difficult to redisperse and become increasingly so upon aging, a process resembling the insolubilization of moist powders. Similarly the conditions that prevail in frozen milk are physico-chemically analogous to those in high moisture milk powder.[456] The caseinate complex in frozen milk is comparably insolubilized without evidence of sugar-protein interaction.

Lactose is actually essential to the stability of the caseinate complex under many conditions, and its crystallization in milk products during frozen storage is highly detrimental to solubility.[397,408] Furthermore, Gerlsma[102] observed that the caseinate complex, substantially freed from lactose by centrifugal separation, loses approximately half of its capacity to redisperse on spray drying. The restoration of lactose, or the addition of glucose, sucrose, or sorbitol effectively protects the solubility of the caseinate during spray drying. Therefore, the crystallization of lactose in high moisture milk powder must exert a more substantial influence on the deterioration of protein solubility, than that attributed merely to its effect on the activity of the water in the system. As a protective factor, the lactose conceivably moderates the destabilizing influence of calcium ions either by direct complex formation[138] or by lowering the ionizing power of the calcium salts in its solutions.[411,412] The concentrated, amorphous lactose matrix in moist milk powder, prior to its crystallization, may further mediate as a physical barrier against micellar aggregation as in frozen concentrated milk, thereby preserving the ability of the colloid to redisperse on reconstitution. Interaction between lactose and casein particularly under conditions involving extensive degradation of the sugar-protein complex, would inevitably supplement the deterioration of casein solubility.

REFERENCES

1. Adachi, S., *Kagaku*, **24**, 261 (1954).
2. Adachi, S., and Patton, S., *J. Dairy Sci.*, **44**, 1375 (1961).
3. Alexander, J., *J. Am. Chem. Soc.*, **32**, 680 (1910).
4. Anson, M. L., "*Advances in Protein Chem.*," **2**, 361 (1945).
5. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiology*, **17**, 399 (1934).
6. Aschaffenburg, R., and Drewry, J., *Nature*, **180**, 376 (1957).
7. Ayers, S. H., **202**, *U.S. Dept. Agr. Bull.*, (1915).
8. Babcock, S. M., 12th Ann. Rept. Wis. Agr. Expt. Sta., p. 93, (1895).
9. Baker, J. M., and Gehrke, C. W., *J. Dairy Sci.*, **39**, 1221 (1956).
10. Ball, C. O., *Food Res.*, **20**, 351 (1955).
11. Bardach, B., *Monatsh*, **18**, 199 (1897).
12. Bate-Smith, E. C., and Hawthorne, J. R., *J. Soc. Chem. Ind.*, **64**, 297 (1945).
13. Belec, J., and Jenness, R., *J. Dairy Sci.*, **44**, 1163 (1961).
14. Bell, R. W., *J. Biol. Chem.*, **64**, 391 (1925).
15. Bell, R. W., Curran, H. R., and Evans, F. R., *J. Dairy Sci.*, **27**, 913 (1944).
16. Bell, R. W., and Webb, B. H., *J. Dairy Sci.*, **26**, 579 (1943).
17. Benton, A. G., *J. Dairy Sci.*, **12**, 481 (1929).
18. Benton, A. G., and Albery, H. G., *J. Biol. Chem.*, **68**, 251 (1926).
19. Bernheim, F., Neurath, H., and Erickson, J. O., *J. Biol. Chem.*, **144**, 259 (1942).
20. Berridge, N. J., *Nature*, **149**, 194 (1942).
21. Berridge, N. J., "*Adv. in Enzymology*," **15**, 423 (1945). (Ed. F. F. Nord, Interscience Publishers, Inc., New York.)
22. Berridge, N. J., "The Enzymes," J. B. Sumner and K. Myrback, Vol. I, Part 2, Chap. 34. Academic Press Inc., New York (**1951**).
23. Bishov, S. J., and Mitchell, J. H., Jr., *Food Tech.*, **10**, 312 (1956).
24. Blair, S., *J. Dairy Res.*, **9**, 347 (1938).
25. Bohren, H. U., and Wenner, V. R., *J. Dairy Sci.*, **44**, 1213 (1961).
26. Boogaerdt, J., *Nature*, **174**, 884 (1954).
27. Boyd, J. M., U.S. Patent 2,827,381, March 18, 1958.
28. Briggs, D. R., and Hull, R., *J. Am. Chem. Soc.*, **67**, 2007 (1945).
29. Brunner, J. R., and Thompson, M. P., *J. Dairy Sci.*, **42**, 1881 (1959).
30. Bull, H. B., and Currie, B. T., *J. Am. Chem. Soc.*, **68**, 742 (1946).
31. Burg, P. van der, *Neth. Milk Dairy J.*, **1**, 69 (1947).
32. Burton, H., *J. Dairy Res.*, **21**, 194 (1954).
33. Burton, H., *J. Dairy Res.*, **22**, 200 (1955).
34. Burton, H., *Dairy Ind.*, **21**, 38 (1956).
35. Burton, H., *J. Dairy Res.*, **23**, 92 (1956).
36. Calbert, H. E.. and Swanson, A. M., *XV Intern. Dairy Congr., London, Proc.*, **1**, 442 (1959).

37. Carr, C. W., *Arch. Biochem. and Biophys.*, **46**, 417 (1953).
38. Carr, R. E., and Trout, G. M., *Food Res.*, **7**, 360 (1942).
39. Cerbulis, J., and Zittle, C. A., *Arch. of Biochem. & Biophys.*, **81**, 418 (1959).
40. Chanutin, A., Ludewig, S., and Masket, A. V., *J. Biol. Chem.*, **143**, 737 (1942).
41. Cherbuliez, E., and Baudet, P., *Helv. Chim. Acta*, **33**, 1673 (1950).
42. Chick, H., and Martin, C. J., *J. Physiol.*, **40**, 404 (1910).
43. Chick, H., and Martin, C. J., *J. Physiol.*, **43**, 1 (1911).
44. Chick, H., and Martin, C. J., *J. Physiol.*, **45**, 61 (1912).
45. Christianson, G., Jenness, R., and Coulter, S. T., *Anal. Chem.*, **26**, 1923 (1954).
46. Christensen, L. K., *Nature*, **163**, 1003 (1949).
47. Cohn, E. J., *J. Gen. Physiol.*, **4**, 697 (1922).
48. Cole, W. C., and Tarassuk, N. P., *J. Dairy Sci.*, **29**, 421 (1946).
49. Coulter, S. T., *J. Dairy Sci.*, **38**, 1180 (1955).
50. Coulter, S. T., Jenness, R., and Crowe, L. K., *J. Dairy Sci.*, **31**, 986 (1948).
51. Coulter, S. T., Jenness, R., and Geddes, W. F., *Adv. Food Research*, **3**, 45 (1951). Ed. E. M. Mrak and G. F. Stewart, Academic Press, Inc., New York.
52. Crammer, J. L., and Neuberger, A., *Biochem. J.*, **37**, 302 (1943).
53. Crossley, E. L., *J. Dairy Res.*, **14**, 160 (1945).
54. Dahlberg, A. O., and Garner, H. S., *U.S. Dept. Agr. Bull.*, **944**, (1921).
55. DellaMonica, E. S., Custer, J. H., and Zittle, C., *J. Dairy Sci.*, **41**, 465 (1958).
56. Davies, D. T., and White, J. C. D., *J. Dairy Res.*, **27**, 171 (1960).
57. Kadt, G. S. de, and van Minnen, G., *Rec. trav. chim.*, **62**, 257 (1943).
58. Deysher, E. F., Webb, B. H., and Holm, G. E., *J. Dairy Sci.*, **12**, 80 (1929).
59. Deysher, E. F., Webb, B. H., and Holm, G. E., *J. Dairy Sci.*, **27**, 345 (1944).
60. Diffloth, P., *Bull, Sci. Pharmacol.*, **10**, 273 (1904).
61. Doan, F. J., *J. Dairy Sci.*, **14**, 527 (1931).
62. Doan, F. J., and Minster, C. H., Pa. Agr. Expt. Sta. Bull., **258**, 28 (1930).
63. Doan, F. J., and Minster, C. H., Pa. Agr. Expt. Sta. Tech. Bull., **287**, (1933).
64. Doan, F. J., and Warren, F. G., *J. Dairy Sci.*, **30**, 837 (1947).
65. Doob, H., Jr., Willmann, A., and Sharp, P. F., *Ind. Eng. Chem.*, **34**, 1460 (1942).
66. Duncombe, E., *J. Dairy Sci.*, **7**, 245 (1924).
67. D'yachenko, P. F., *Voprosy Pitaniya*, **12**, No. 3, 65 (1953).
68. D'yachenko, P. F., and Vlodavets, I. N., *Kolloid. Zh.*, **14**, 338 (1952).
69. D'yachenko, P. F., Vlodavets, I. N., and Bogomolova, E., *Molochnaya Prom.*, **14**, No. 6, 33 (1953).
70. Echenique, L., and Suarez, B., *Compt. Rend. Soc. Biol.*, **120**, 570 (1935).
71. Eckles, C. H., and Palmer, L. S., *J. Biol. Chem.*, **27**, 313 (1916).
72. Edkins, J. S., *J. Physiol.*, **12**, 193 (1891).
73. Edmondson, L. F., *J. Dairy Sci.*, **41**, 553 (1958).
74. Edmondson, L. F., *J. Dairy Sci.*, **42**, 910 (1959).
75. Edmondson, L. F., and Tarassuk, N. P., *J. Dairy Sci.*, **39**, 36 (1956).
76. Edmondson, L. F., and Tarussuk, N. P., *J. Dairy Sci.*, **39**, 123 (1956).
77. Eilers, H., Burger, A., Holtkamp, A. G., Mul, H., Vlaming, A. de, and Zwolsman, A. A., *Versas. Landbouwk. Onderzoekingen*, **50**, No. 15G, 1007 (1945).
78. Eilers, H., Saal, R. N. J., and Waarden, M. van der, "Chemical and Physical Investigations on Dairy Products," Elsevier Book Co., New York (**1947**).
79. Ellertson, M. E., and Pearce, S. J., U.S. Patent 2,822,277, February 4, 1958.
80. Emmons, D. B., Price, W. V., and Swanson, A. M., *J. Dairy Sci.*, **42**, 589 (1959).
81. Emmons, D. B., Ph.D. Thesis, Univ. of Wisconsin, Madison (**1957**).
82. Evenhuis, N., *Neth. Milk Dairy J.*, **11**, 225 (1957).
83. Evenhuis, N., and Vries, T. R. de, *Neth. Milk and Dairy J.*, **9**, 146 (1955).
84. Evenhuis, N., and Vries, T. R. de, *Neth. Milk and Dairy J.*, **10**, 1 (1956).
85. Evenhuis, N., and Vries, T. R. de, *Neth. Milk and Dairy J.*, **10**, 101 (1956).
86. Evenhuis, N., and Vries, T. R. de, *Neth. Milk and Dairy J.*, **10**, 180 (1956).
87. Eyring, H., and Stearn, A. E., *Chem. Rev.*, **24**, 253 (1939).
88. Fenger, F., *J. Am. Chem. Soc.*, **45**, 249 (1923).
89. Fitzpatrick, M. M., and Sullivan, R. A., *J. Dairy Sci.*, **40**, 1262 (1957).
90. Food and Drug Admin., *Fed. Register*, **5**, 2442 (July 2, 1940).
91. Ford, T. F., and Ramsdell, G. A., *XII Intern. Dairy Congr. (Stockholm), Proc.*, **2**, 17 (1949).
92. Fouassier, M., *Le Lait*, **4**, 366 (1924).
93. Fox, K. K., Ph.D. thesis, Ohio State Univ., Columbus (1956).
94. Fox, K. K., Holsinger, V. H., Caha, J., and Pallansch, M. J., *J. Dairy Sci.*, **43**, 1396 (1960).
95. Fox, K. K., Van Winkle, Q., Gould, I. A., and Slatter, W. L., *Q. M. Food & Cont. Inst., Ser. I. Food Stability*, No. **6**, Dry Milk Products, p. 66, 1955.
96. Fraenkel-Conrat, J., Cook, B. B., and Morgan, A. F., *Arch. Biochem. Biophys.*, **35**, 157 (1952).
97. Fram, H., *J. Dairy Sci.*, **40**, 19 (1957).
98. Freudenreich, E. V., *Rev. Gen. Lait.*, **4**, 433 (1905).
99. Fuller, R. A., Ph.D. Thesis, Univ. of Minn., Minneapolis (**1955**).
100. Gammack, D. B., and Weckel, K. G., *J. Dairy Sci.*, **40**, 1570 (1957).
101. Garnier, J., *Biochim. and Biophys. Acta*, **38**, 246 (1960).
102. Gerlsma, S. Y., *Neth. Milk and Dairy J.*, **11**, 83 (1957).
103. Giroux, R. N., Calbert, H. E., and Swanson, A. M., *J. Dairy Sci.*, **41**, 710 (1958).

104. Golub, M. A., and Pickett, E. E., *J. Polym. Sci.*, **13**, 427 (1954).
105. Gordon, W. G., Semmett, W. F., Cable, R. S., and Morris, M., *J. Am. Chem. Soc.*, **71**, 3293 (1949).
106. Gorini, L., *Biochim. et Biophys. Acta*, **7**, 318 (1951).
107. Gough, P., and Jenness, R., *J. Dairy Sci.*, **44**, 1163 (1961).
108. Gould, I. A., *J. Dairy Sci.*, **28**, 379 (1945).
109. Gould, I. A., and Frantz, R. S., *J. Dairy Sci.*, **29**, 27 (1946).
110. Gould, I. A., Weaver, E., and Frantz, R. S., *J. Dairy Sci.*, **29**, 33 (1946).
111. Goulden, J. D. S., *Nature, London*, **177**, 85 (1956).
112. Greenbank, G. R., Steinbarger, M. C., Deysher, E. F., and Holm, G. E., *J. Dairy Sci.*, **10** 335 (1927).
113. Grimbleby, F. H., *J. Dairy Res.*, **21**, 207 (1954).
114. Grosser, P., *Biochem. Z.*, **48**, 427 (1913).
115. Groves, M. L., Hipp, N. J., and McMeekin, T. L., *J. Am. Chem. Soc.*, **73**, 2790 (1951).
116. Habermann, W., Mattenheimer, H., Sky-Peck, H., and Sinohara, H., *Chimia (Switz.)*, **15**, 339 (1961).
117. Halwer, M., *Arch. of Biochem. and Biophys.*, **51**, 79 (1954).
118. Halwer, M., Nutting, G. C., and Brice, B. A., *J. Am. Chem. Soc.*, **73**, 2786 (1951).
119. Hankinson, C. L., and Briggs, D. R., *J. Phys. Chem.*, **45**, 943 (1941).
120. Hankinson, C. L., and Palmer, L. S., *J. Dairy Sci.*, **26**, 1043 (1943).
121. Hannan, R. S., and Lea, C. H., *Nature*, **168**, 744 (1951).
122. Hannan, R. S., and Lea, C. H., *Biochim. et Biophys. Acta.*, **9**, 293 (1952).
123. Harden, A., and Macallum, A. B., *Biochem. J.*, **8**, 90 (1914).
124. Harland, H. A., and Ashworth, U.S., *J. Dairy Sci.*, **28**, 879 (1945).
125. Harland, H. A., Coulter, S. T., and Jenness, R., *J. Dairy Sci.*, **32**, 334 (1949).
126. Harland, H. A., Coulter, S. T., and Jenness, R., *J. Dairy Sci.*, **35**, 363 (1952).
127. Harland, H. A., Coulter, S. T., and Jenness, R., *J. Dairy Sci.*, **38**, 858 (1955).
128. Harland, H. A. Coulter, S. T., Townley, V. H., and Jenness, R., *J. Dairy Sci.*, **36**, 568 (1953).
129. Haugaard, G., and Tumerman, L., *Arch. of Biochem. and Biophys.*, **65**, 86 (1956).
130. Haugaard, G., Tumerman, L., and Silvestri, H., *J. Am. Chem. Soc.*, **73**, 4594 (1951).
131. Haurowitz, F., *Kolloid Z.*, **71**, 198 (1935).
132. Haurowitz, F., *Kolloid Z.*, **74**, 208 (1936).
133. Haurowitz, F., Tunca, M., Schwerin, P., and Goksu, V., *J. Biol. Chem.*, **157**, 621 (1945).
134. Heidelberger, M. Davis, B., and Treffers, H. P., *J. Am. Chem. Soc.*, **63**, 498 (1941).
135. Henry, K. M., Kon, S. K., Lea, C. H., and Smith, J. D. H., *12th Intern. Dairy Congr. (Stockholm) Proc.*, **2**, 166 (1949).
136. Henry, K. M., Kon, S. K., Lea, C. H., and White, J. C. D., *J. Dairy Res.*, **15**, 292 (1948).
137. Herreid, E. O., Rept. Proc. Conference on Milk Concentrates, *Eastern Utilization and Research Div., U.S. Dept. Agr.*, Philadelphia, p. 38 (1955).
138. Herrington, B.L., *J. Dairy Sci.*, **17**, 805 (1934).
139. Herrington, B. L., "Milk and Milk Processing," McGraw-Hill Book Co., New York (**1948**).
140. Hetrick, J. H., and Tracy, P. H., *J. Dairy Sci.*, **31**, 867 (1948).
141. Hetrick, J. H., and Tracy, P. H., *J. Dairy Sci.*, **33**, 410 (1950).
142. Heymann, E., *Biochem. J.*, **30**, 127 (1936).
143. Heyndrick, G. V., and Vleeschauwer, A. de, *Experientia*, **8**, 317 (1952).
144. Hilgeman, M., and Jenness, R., *J. Dairy Sci.*, **34**, 483 (1951).
145. Hipp, N. J., Groves, M. L., Custer, J. H., and McMeekin, T. L., *J. Am. Chem. Soc.*, **72**, 4928 (1950).
146. Hipp, N. J., Groves, M. L., Custer, J. H., and McMeekin, T. L., *J. Dairy Sci.*, **35**, 272 (1952).
147. Hipp, N. J., Groves, M. L., and McMeekin, T. L., *J. Am. Chem. Soc.*, **74**, 4822 (1952).
148. Hipp, N. J., Groves, M. L., and McMeekin, T. L., *J. Am. Chem. Soc.*, **79**, 2559 (1957).
149. Hodge, J. E., *J. Agr. Food Chem.*, **1**, 928 (1953).
150. Hodge, J. E., and Rist, C. E., *J. Am. Chem. Soc.*, **75**, 316 (1953).
151. Hoff, J. E., Sunyach, J., Proctor, B. E., and Goldblith, S. A., *Food Tech.*, **14**, 24 (1960).
152. Hoff, J. E., Sunyach, J., Proctor, B. E., and Goldblith, S. A., *Food Tech.*, **14**, 27 (1960).
153. Hoff, J. E., Wertheim, J. H., Roychoudhury, R. N., Deolalkar, S. T., Proctor, B. E., and Goldblith, S. A., *Food Tech.*, **12**, 648 (1958).
154. Hofman, T., *Biochem. J.*, **69**, 139 (1958).
155. Hollender, H., and Weckel, K. G., *Food Res.*, **6**, 335 (1941).
156. Holm, G. E., Cited in Ph.D. Thesis, B. H. Webb, Cornell University, Ithaca, (**1931**.)
157. Holm, G. E., Deysher, E. F., and Evans, F. R., *J. Dairy Sci.*, **6**, 556 (1923).
158. Holm, G. E., Webb, B. H., and Deysher, E. F., *J. Dairy Sci.*, **15**, 331 (1932).
159. Holter, H., *Biochem. Z.*, **255**, 160 (1932).
160. Holter, H., and Li, S. O., *Acta Chem. Scand.*, **4**, 1321 (1950).
161. Hopkins, F. G., *Nature*, **126**, 328 (1930).
162. Hostettler, H., and Imhof, K., *Milchwissenschaft*, **6**, 351, 400 (1951).
163. Hostettler, H., and Imhof, K., *Landwirtsch, Jahrb. d. Schweiz*, **66**, 307 (1952).
164. Hostettler, H., and Imhof, K., *13th Intern. Dairy Congr. (Hague), Proc.*, **2**, 423 (1953).
165. Hostettler, H., and Rychener, E., *12th Intern. Dairy Congr. (Stockholm), Proc.*, **2**, 175 (1949).
166. Hostettler, H., and Stein, J., *Landwertsch. Jahrb. Schweiz N. S.*, **7**, 163 (1958).
167. Hostettler, H., Stein, J., and Bruderer, G., *Landwertsch. Jahrb. Schweiz*, **71**, No. 2: 143 (1957).

168. Howat, G. R., Smith, J. A. B., Waite, R., and Wright, N. C., *J. Dairy Res.*, **10,** 498 (1939).
169. Howat, G. R., and Wright, N. C., *J. Dairy Res.*, **4,** 265 (1933).
170. Howat, G. R., and Wright, N. C., *J. Dairy Res.*, **5,** 236 (1934).
171. Howat, G. R., and Wright, N. C., *Biochem. J.*, **28,** 1336 (1934).
172. Howat, G. R., and Wright, N. C., *Biochem. J.*, **30,** 1413 (1936).
173. Hsu, R. Y. H., Anderson, L., Baldwin, R. L., Ernstrom, C. A., and Swanson, A. M., *Nature*, **182,** 798 (1958).
174. Huggins, C., Tapley, D. F., and Jensen, E. V., *Nature*, **167,** 592 (1951).
175. Humbert, E. S., Brunner, J. R., and Trout, G. M., *Food Tech.*, **10,** 134 (1956).
176. Hutton, J. T., and Patton, S., *J. Dairy Sci.*, **35,** 699 (1952).
177. Iacobellis, M., *Arch. Biochem. and Biophys.*, **59,** 199 (1955).
178. Jennings, R. K., and Smith, L. D., *J. Immunol.*, **45,** 105 (1942).
179. Jenness, R., Paper presented at 47th Ann. Mtg. *Am. Dairy Sci. Assn.*, Davis, Calif. (1952). Abst. M14, J. Dairy Sci., **35,** 482 (1952).
180. Jenness, R., *J. Agr. Food Chem.*, **2,** 75 (1954).
181. Jenness, R., and Coulter, S. T., *Quartermaster Food and Container Institute Surveys of Prog. on Military Subsistence. Problems Ser. I, Food Stability No.*, **6,** Dry Milk Products, p. 17 (1955).
182. Jolles, P., and Alais, C., *Acad. Sci., Compt. Rend.*, **251,** 2605 (1960).
183. Jolles, P., Alais, C., Jolles, J., *Biochim. et Biophys. Acta*, **5,** 309, 315 (1961).
184. Josephson, D. V., and Reeves, C. B., *J. Dairy Sci.*, **30,** 737 (1947).
185. Kadaba, L. R., Ph.D. Thesis, Univ. of Illinois, Urbana (**1957**).
186. Kalan, E. B., and Telka, M., *Arch. Biochem. and Biophys.*, **79,** 275 (1959).
187. Kannan, A., and Jenness, R., *J. Dairy Sci.*, **39,** 911 (1956).
188. Kass, J. P., and Palmer, L. S., *Ind. Eng. Chem.*, **32,** 1360 (1940).
189. Katchalsky, A., *Biochem. J.*, **35,** 1024 (1941).
190. Katchalsky, A., and Sharon, N., *Biochim. et Biophys. Acta*, **10,** 290 (1953).
191. Kauzmann, W., and Douglas, R. G., Jr., *Arch. Biochem. and Biophys.*, **65,** 106 (1956).
192. Keeney, P. G., Patton, S., and Josephson, D. V., *J. Dairy Sci.*, **33,** 526, (1950).
193. Keeney, M., and Josephson, D. V., *J. Dairy Sci.*, **30,** 539 (1947).
194. Kern, J., Weiser, H. H., Harper, W. J., and Gould, I. A., *J. Dairy Sci.*, **37,** 904 (1954).
195. Kieferle, F., and Gloetzl, J., *Milchw. Forsch.*, **11,** 62 (1930).
196. King, N., *12th Intern. Dairy Congr.* (*Stockholm*), *Proc.*, **2,** 36 (1949).
197. Kitzes, A. S., Ph.D. thesis, Univ. of Minnesota, Minneapolis (**1948**).
198. Kline, R. W., and Stewart, G. F., *Ind. Eng. Chem.*, **40,** 919 (1948).
199. Kometiani, P. A., *Milchw. Forsch.*, **12,** 433 (1931).
200. Kondo, M. J., *J. Home Econ.* (*Japan*), **1,** No. 1, 14 (1951).
201. Kondo, M., *J. Home Econ.* (*Japan*), **3,** No. 2, 1 (1953).
202. Krejci, L. E., Jennings, R. K., and Smith, L. D., *J. Immunol.*, **45,** 111 (1942).
203. Kuntzel, A., and Doehner, K., *Kolloid-Beihefte*, **51,** 277 (1940).
204. Lampitt, L. H., and Bushill, J. H., *Analyst*, **56,** 778 (1931).
205. Larson, B. L., and Jenness, R., *J. Dairy Sci.*, **33,** 896 (1950).
206. Larson, B. L., and Jenness, R., *J. Am. Chem. Soc.*, **74,** 3090 (1952).
207. Larson, B. L., Jenness, R., Geddes, W. F., and Coulter, S. T., *Cereal Chem.*, **28,** 351 (1951).
208. Larson, B. L., Jenness, R., and Geddes, W. F., *Cereal Chem.*, **29,** 440 (1952).
209. Larson, B. L., and Rolleri, G. D., *J. Dairy Sci.*, **38,** 351 (1955).
210. Larsen, R. A., Jenness, R., and Geddes, W. F., *Cereal Chem.*, **26,** 189 (1949).
211. Lea, C. H., and Hannan, R. S., *Biochim. et Biophys. Acta*, **3,** 313 (1949).
212. Lea, C. H., *J. Dairy Res*, **15,** 369 (1948).
213. Lea, C. H., and Hannan, R. S., *Biochim. et Biophys. Acta*, **4,** 518 (1950).
214. Lea, C. H., and Hannan, R. S., *Biochim. et Biophys. Acta*, **5,** 433 (1950).
215. Lea, C. H., and Hannan, R. S., *Nature*, **165,** 438 (1950).
216. Lear, S. A., and Foster, H. G., *J. Dairy Sci.*, **32,** 509 (1949).
217. Leighton, A., and Deysher, E. F., World's Dairy Congress, Syracuse Proc., **2,** 1276 (1924).
218. Leighton, A., and Mudge, C. S., *J. Biol. Chem.*, **56,** 53 (1923).
219. Leviton, A., U.S.D.A., Wash., D.C. Unpublished results (1961).
220. Leviton, A., and Pallansch, M. J., *J. Dairy Sci.*, **44,** 633 (1961).
221. Leviton, A., Pallansch, M. J., and Webb, B. H., *16th International Dairy Congr. Proc.*, B: Sec. V, **2.** 1009 Copenhagen (1962).
222. Lewis, V. M., and Lea, C. H., *Biochim. et Biophys. Acta*, **4,** 532 (1950).
223. Libbey, L. M., and Ashworth, U.S., *J. Dairy Sci.*, **44,** 1016 (1961).
224. Linderstrom-Lang, K., *Compt. rend. trav. lab. Carlsberg*, **17,** 149 (1929).
225. Linderstrom-Lang, K., Hotchkiss, R. D., and Johansen, G., *Nature*, **142,** 996 (1938).
226. Linderstrom-Lang, K., and Kodama, S., *Compt. rend. trav. lab. Carlsberg*, **16,** No. 1 (1925).
227. Lindet, L., *Bull. Soc. Chim.*, Ser. 4, **13,** 929 (1913).
228. Lineweaver, H., and Hoover, S. R., *J. Biol. Chem.*, **137,** 325 (1941).
229. Litman, I. I., and Ashworth, U.S., *J. Dairy Sci.*, **40,** 403 (1957).
230. Loeb, J., *J. Gen. Phys.*, **3,** 547 (1921).
231. Loeb, J., *J. Gen. Phys.*, **4,** 73 (1921).
232. Loeb, J., *J. Am. Chem. Soc.*, **44,** 1930 (1922).
233. Loeb, J. and Loeb, R. F., *J. Gen. Phys.*, **4,** 187 (1921).
234. Long, J. E., Ph.D. Thesis, Ohio State Univ., Columbus (**1958**).

235. Long, J., Van Winkle, Q., and Gould, I. A., *J. Dairy Sci.*, **41**, 317 (1958).
236. Lumry, R., and Eyring, H., *J. Phys. Chem.*, **58**, 110 (1954).
237. MacRae, H. F., and Baker, B. E., *J. Dairy Sci.*, **41**, 233 (1958).
238. Magee, H. E., and Harvey, D., *Biochem. J.*, **20**, 873 (1926).
239. Malpress, F. H., *Biochem. J.*, **80**, 19P (1961).
240. Manus, L. J., and Ashworth, U. S., *J. Dairy Sci.*, **31**, 935 (1948).
241. Marshall, W. R., Jr., *Quartermaster Food and Container Institute Surveys of Progr. on Military Subsistence, Problems Ser. 1. Food Stability No. 6*, Dry Milk Products *Symposium*, p. 90 (**1955**).
242. Mattenheimer, H., Nitschmann, H., and Zahler, P., *Helv. Chim. Acta*, **35**, 1970 (1952).
243. Mattick, E. C. V., and Hallett, H. S., *J. Agric. Sci.*, **19**, 452 (1929).
244. Maurer, P. H., and Heidelberger, M., *J. Am. Chem. Soc.*, **73**, 2070 (1951).
245. Mauron, J., Mottu, F., Bujard, E., and Egli, R. H., *Arch. Biochem. and Biophys.*, **59**, 433 (1955).
246. Maxcy, R. B., and Sommer, H. H., *J. Dairy Sci.*, **33**, 407 (1950).
247. Maxcy, R. B., and Sommer, H. H., *J. Dairy Sci.*, **37**, 60 (1954).
248. Maxcy, R. B., and Sommer, H. H., *J. Dairy Sci.*, **37**, 306 (1954).
249. Maxcy, R. B., and Sommer, H. H., *J. Dairy Sci.*, **37**, 1061 (1954).
250. McGugan, W. A., Zehren, V. F., Zehren, V. L., and Swanson, A. M., *Science*, **120**, 435 (1954).
251. McInerney, T. J., *J. Dairy Sci.*, **3**, 220 (1920).
252. McMeekin, T. L., *J. Milk and Food Technol.*, **15**, 57 (1952).
253. McMeekin, T. L., Groves, M. L., and Hipp, N. J., *131st Meeting Am. Chem. Soc., Abst. of papers*, p. 65C, Abstr. **143**, April, 1957.
254. McMeekin, T. L., Hipp, N. J., and Groves, M. L., *Arch. Biochem. and Biophys.*, **83**, 35 (1959).
255. McMeekin, T. L., and Warner, R. C., *J. Am. Chem. Soc.*, **64**, 2393 (1942).
256. Mellander, O., *Biochem. Z.*, **300**, 240 (1939).
257. Mellon, E. F., Korn, A. H., and Hoover, S. R., *J. Am. Chem. Soc.*, **69**, 827 (1947).
258. Mellon, E. F., Korn, A. H., and Hoover, S. R., *J. Am. Chem. Soc.*, **70**, 3040 (1948).
259. Menefee, S. G., Overman, O. R., and Tracy, P. H., *J. Dairy Sci.*, **24**, 953 (1941).
260. Miller, P. G., and Sommer, H. H., *J. Dairy Sci.*, **23**, 405 (1940).
261. Milroy, T. H., *Biochem. J.*, **9**, 215 (1915).
262. Mirsky, A. E., and Pauling, L., *Proc. Nat'l. Acad. Sci. U. S.*, **22**, 439 (1936).
263. Mohammed, A., Fraénkel-Conrat, H., and Olcott, H. S., *Arch. Biochem.*, **24**, 157 (1949).
264. Moir, G. M., *J. Dairy Research*, **2**, 68 (1930).
265. Moir, G. M., *J. Dairy Research*, **3**, 80 (1931).
266. Morr, C. V., Gould, I. A., and Harper, W. J., *J. Dairy Sci.*, **38**, 594 (1955).
267. Morr, C. V., Harper, W. J., and Gould, I. A., *J. Dairy Sci.*, **40**, 964 (1957).
268. Mojonnier, T., and Troy, H. C., "The Technical Control of Dairy Products," Caspar, Krueger, Dory, Co., Milwaukee (**1926**).
269. Morris, H. A., Coulter, S. T., Combs, W. B., and Heinzel, L. R., *J. Dairy Sci.*, **34**, 487 (1951).
270. Moster, J. B., and Chapman, R. A., *Can. J. Research*, **27F**, 429 (1949).
271. Moyer, L. S., *J. Phys. Chem.*, **42**, 71 (1938).
272. Moyer, L. S., *J. Biol. Chem.*, **133**, 29 (1940).
273. Mularz, V., and Swanson, A. M., *J. Dairy Sci.*, **36**, 569 (1953).
274. Murthy, L., Herreid, E. O., and Whitney, R. McL., *J. Dairy Sci.*, **41**, 1324 (1958).
275. Nelson, V., *J. Dairy Sci.*, **31**, 415 (1948).
276. Nelson, V., *J. Dairy Sci.*, **32**, 775 (1949).
277. Nelson, V., *J. Dairy Sci.*, **35**, 388 (1952).
278. Neurath, H., Greenstein, J. P., Putnam, F. W., and Erickson, J. O., *Chem. Rev.*, **34**, 157 (1944).
279. Neurath, H., and Saum, A. M., *J. Biol. Chem.*, **128**, 347 (1939).
280. Nichols, J. B., Bailey, E. D., Holm, G. E., Greenbank, G. R., and Deysher, E. F., *J. Phys. Chem.*, **35**, 1303 (1931).
281. Nitschmann, H., *Helv. Chim. Acta*, **32**, 1258 (1949).
282. Nitschmann, H., and Henzi, R., *Helv. Chim. Acta*, **42**, 1985 (1959).
283. Nitschmann, H., and Lehmann, W., *Experientia*, **3**, 153 (1947).
284. Nitschmann, H., and Lehmann, W., *Helv. Chim. Acta*, **30**, 804 (1947).
285. Nitschmann, H., and Varin, R., *Helv. Chim. Acta*, **34**, 1421 (1951).
286. Nitschmann, H., Wissmann, H., and Henzi, R., *Chimia*, **11**, 76 (1957).
287. Oeda, M., *Dairy Sci. Abst.*, **22**, 638 (1960).
288. Ogston, A. G., and Tombs, M. P., *Biochem. J.*, **66**, 399 (1957).
289. Okuda, Y., and Zoller, H. F., *J. Ind. Eng. Chem.*, **13**, 515 (1921).
290. Orla-Jensen, S., and Plattner, E., *Rev. Gen. Lait*, **4**, 361 (1905).
291. Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, **33**, 243 (1918).
292. Otting, H. E., U.S. Patent 2,490,599, December 6, 1949.
293. Otting, H. E., Chrysler, L. H., and Almy, E. F., U.S. Patent 2,473,493, June 14, 1949.
294. Palmer, L. S., *Proc. Soc. Exp. Biol. Med.*, **19**, 137 (1921).
295. Palmer, L. S., and Eckles, C. H., *Am. J. Diseases Child.*, **13**, 413 (1917).
296. Patton, S., *J. Dairy Sci.*, **33**, 102 (1950).
297. Patton, S., *J. Dairy Sci.*, **33**, 324 (1950).
298. Patton, S., *J. Dairy Sci.*, **33**, 904 (1950).
299. Patton, S., *J. Dairy Sci.*, **35**, 1053 (1952).
300. Patton, S., *J. Dairy Sci.*, **38**, 457 (1955).
301. Patton, S., and Flipse, R. J., *J. Dairy Sci.*, **36**, 766 (1953).
302. Patton, S., and Flipse, R. J., *Science*, **125**, 1087 (1957).

303. Patton, S., Hill, E. G., and Foreman, E. M., *Science*, **107**, 623 (1948).
304. Patton, S., and Josephson, D. V., *J. Dairy Sci.*, **32**, 222 (1949).
305. Patton, S., and Josephson, D. V., *J. Dairy Sci.*, **32**, 398 (1949).
306. Patton, S., Keeney, P. G., and Potter, F. E., *Quartmaster Food and Container Inst. Surveys of Progr. on Military Subsistence Problems, Ser. J., Food Stability No. 6* Dry Milk Products, p. 44 (**1955**).
307. Patton, S., Salander, R. C., and Piano, M., *Food Res.*, **19**, 444 (1954).
308. Payens, T. A. J., *Nature*, **181**, **114**, (1958).
309. Pech, A. E., U.S. patent 2,892,723, June 30, 1959.
310. Peebles, D. D., U.S. patent 2,565,085, August 21, 1951.
311. Pedersen, K. O., *Nature*, **128**, **150** (1931).
312. Perlmann, G. E., *Disc. Faraday Soc.*, **13**, 67 (1953).
313. Perlmann, G., *Adv. Enzymology*, **10**, 1 (1955).
314. Peterson, R. F., Nauman, L. W., and McMeekin, T. L., *J. Am. Chem. Soc.*, **80**, 95 (1958).
315. Philpot, J. St. L., *Nature, Lond.*, **142**, 1024 (1938).
316. Philpot, F. J., and Philpot, J. St. L., *Proc. Roy. Soc. A*, **170**, 60 (1939).
317. Polis, B. D., Schmukler, H. W., Custer, J. H., and McMeekin, T. L., *J. Am. Chem. Soc.*, **72**, 4965 (1950).
318. Powell, M. E., *J. Dairy Sci.*, **19**, 305 (1936).
319. Powell, M. E., and Palmer, L. S., *J. Dairy Sci.*, **18**, 401 (1935).
320. Putnam, F. W., "The Proteins" [H. Neurath and K. Bailey, eds., Vol. I, Part B, p. 807, Academic Press, N.Y., **1953**].
321. Putnam, F. W., and Neurath, H., *J. Biol. Chem.*, **160**, 239 (1945).
322. Pyenson, H., and Dahle, C. D., *J. Dairy Sci.*, **21**, 169, 407 (1938).
323. Pyne, G. T., *Biochem. J.*, **26**, **1006** (1932).
324. Pyne, G. T., *Biochem. J.*, **39**, **385** (1945).
325. Pyne, G. T., *12th Int. Dairy Congr.* (*Stockholm*), *Proc.*, **2**, 229 (1949).
326. Pyne, G. T., *Chem. and Ind.*, **b.**, 302 (**1953**).
327. Pyne, G. T., *13th Intern. Dairy Congr.* (*The Hague*), *Proc.*, **3**, 1032 (1953).
328. Pyne, G. T., *Dairy Sci. Abst.*, **17**, 531 (1955).
329. Pyne, G. T., and McGann, T. C. A., *J. Dairy Res.*, **27**, 9 (1960).
330. Pyne, G. T., and McHenry, K. A., *J. Dairy Res.*, **22**, 60 (1955).
331. Pyne, G. T., and Ryan, J. J., *J. Dairy Res.*, **17**, 200 (1950).
332. Ramsdell, G. A., and Hufnagel, C. A., *13th Intern. Dairy Congr.* (*The Hague*) *Proc.*, **3**, 1025 (1953).
333. Ramsdell, G. A., Johnson, W. T., Jr., and Evans, F. R., *J. Dairy Sci.*, **14**, 93 (1931).
334. Ramsdell, G. A., and Whittier, E. O., *J. Dairy Sci.*, **36**, 437 (1953).
335. Ramsdell, G. A., and Whittier, E. O., *J. Biol. Chem.*, **154**, 413 (1944).
336. Ramsey, R. J., Tracy, P. H., and Ruehe, H. A., *J. Dairy Sci.*, **16**, 17 (1933).
337. Reisfeld, R. A., Ph.D. Thesis, Ohio State Univ., Columbus (**1957**).
338. Reyniers, J. A., U.S. patent 2,518,493, Aug. 15, 1950.
339. Rice, F. E., and Markley, A. L., *J. Dairy Sci.*, **7**, 468 (1924).
340. Rimington, C., *Biochem. J.*, **21**, 272 (1927).
341. Rimington, C., and Kay, H. D., *Biochem. J.*, **20**, 777 (1926).
342. Roeder, G., *13th Intern. Dairy Congr.* (*Hague*), *Proc.*, **2**, 312 (1953).
343. Rogers, L. A., Deysher, E. F., and Evans, F. R., *J. Dairy Sci.*, **3**, 468 (1920).
344. Rogers, L. A., Deysher, E. F., and Evans, F. R., *J. Dairy Sci.*, **4**, 294 (1921).
345. Rose, D., *J. Dairy Sci.*, **44**, 430 (1961).
346. Rose, D., *J. Dairy Sci.*, **44**, 1405 (1961).
347. Rose, D., *J. Dairy Sci.*, **44**, 1763 (1961).
348. Rose, D., and Tessier, H., *J. Dairy Sci.*, **42**, 969 (1959).
349. Rowland, S. J., *J. Dairy Res.*, **5**, 46 (1933).
350. Rowland, S. J., *J. Dairy Res.*, **8**, 1 (1937).
351. Rowland, S. J., *J. Dairy Res.*, **8**, 6 (1937).
352. Rupp, P., U.S. Dept. Agr., Bur. An. Ind. *Bull.*, **166**, (1913).
353. Sandelin, A. E., *Molkereiwiss. Z.*, **1**, (1943).
354. Sandelin, A. E., *Meijerit. Aikakausk*, **5**, 1 (1943).
355. Sanders, G. P., and Sager, O. S., *J. Dairy Sci.*, **31**, 845 (1948).
356. Scheraga, H. A., and Mandelkern, L., *J. Am. Chem. Soc.*, **75**, 179 (1953).
357. Schober, R., and Hetzel, H., *Dairy Sci. Abst.*, **17**, 862 (1955).
358. Schroeder, L. J., Iacobellis, M., and Smith, A. H., *J. Biol. Chem.*, **212**, 973 (1955).
359. Seehafer, M. E., and Swanson, A. M., *J. Dairy Sci.*, **43**, 846 (1960).
360. Seekles, L., and Smeets, W. T. G. M., *Neth. Milk and Dairy J.*, **1**, 7 (1947).
361. Seekles, L., and Smeets, W. T. G. M., *Lait*, **34**, 610 (1954).
362. Senti, F. R., and Warner, R. C., *J. Am. Chem. Soc.*, **70**, 3318 (1948).
363. Shahani, K. M., and Sommer, H. H., *J. Dairy Sci.*, **34**, 1035 (1951).
364. Shulman, S., and Ferry, J. D., *J. Phys. and Colloid Chem.*, **55**, 135 (1951).
365. Simonson, H. D., and Tarassuk, N. P., *J. Dairy Sci.*, **35**, 166 (1952).
366. Slatter, W. L., and Van Winkle, Q., *J. Dairy Sci.*, **35**, 1083 (1952).
367. Smeets, W. T. G. M., *Neth. Milk Dairy J.*, **9**, 249 (1955).
367A. Smeets, W. T. G. M., and Seekles, L., *Nature*, **169**, 802 (1952).
368. Söldner, F., *Landw. Versucht.*, **35**, 351 (1888).

369. Sommer, H. H., "Market Milk and Related Products," 2nd Ed. Published by the author Madison (**1946**).
370. Sommer, H. H., and Binney, T. H., *J. Dairy Sci.*, **6**, 176 (1923).
371. Sommer, H. H., and Hart, E. B., *J. Biol. Chem.* **35**, 313 (1918).
372. Sommer, H. H., and Hart, E. B., *J. Biol. Chem.*, **40**, 137 (1919).
373. Sommer, H. H., and Hart, E. B., *Wisc. Agr. Expt. Sta. Res. Bull.*, **67**, (1926).
374. Spellacy, J. R., "Casein, Dried and Condensed Whey," Lithotype Process Co., San Francisco (**1953**).
375. Stebnitz, V. C., and Sommer, H. H., *J. Dairy Sci.*, **18**, 805 (1935).
376. Stebnitz, V. C., and Sommer, H. H., *J. Dairy Sci.*, **19**, 101 (1936).
377. Stewart, A. P., Jr., Johnson, R. A., and Anderson, P. T., U.S. Patent 3,008,840, Nov. 14, 1961.
378. Stewart, A. P., Jr., Johnson, R. A., and Wilcox, D. F., U.S. Patent 2,886,450, May 12, 1959.
379. Stewart, G. F., Best, L. R., and Lowe, B., *Proc. Inst. Food Technol.*, **4**, 77 (1943).
380. Storrs, A. B., *J. Dairy Sci.*, **25**, 19 (1942).
381. Sullivan, R. A., Fitzpatrick, M. M., and Stanton, E. K., *Nature*, **183**, 616 (1959).
382. Sullivan, R. A., Fitzpatrick, M. M., Stanton, E. K., Annino, R., Kissel, G., and Palermiti, F., *Arch. Biochem., and Biophys.*, **55**, 455 (1955).
383. Sullivan, R. A., Hollis, R. A., and Stanton, E. K., *J. Dairy Sci.*, **40**, 830 (1957).
384. Sundararajan, T. A., and Sarma, P. S., *Biochem. J.*, **65**, 261 (1957).
385. Supplee, G. C., and Bellis, B., *J. Dairy Sci.*, **8**, 39 (1925).
386. Sutermeister, E., and Browne, F. L., "Casein and Its Industrial Applications" ACS monograph, No. 30, Reinhold Publishing Corp., New York (**1939**).
387. Svedberg, T., *Kolloid, Z.*, **51**, 10 (1930).
388. Svedberg, T., Carpenter, L. M., and Carpenter, D. C., *J. Am. Chem. Soc.*, **52**, 701 (1930).
389. Tamsma, A. F., and Tarassuk, N. P., *J. Dairy Sci.*, **39**, 26 (1956).
390. Tarassuk, N. P., and Nury, M. S., *J. Dairy Sci.*, **35**, 857 (1952).
391. Tarassuk, N. P., and Tamsma, A. F., *J. Agr. and Food Chem.*, **4**, 1033 (1956).
392. Tauber, H., and Kleiner, I. S., *J. Biol. Chem.*, **96**, 745, 755 (1932).
393. Ter Horst, M. G., *Neth. Milk Dairy J.*, **1**, 137 (1947).
394. Ter Horst, M. G., *Neth. Milk Dairy J.*, **4**, 246 (1950).
395. Tessier, H., and Rose, D., *Can. J. Technol.*, **34**, 211 (1956).
396. Tessier, H., and Rose, D., *J. Dairy Sci.*, **44**, 1238 (1961).
397. Tessier, H., Rose, D., and Lusena, C. V., *Canad. J. Tech.*, **34**, 131 (1956).
398. Thurston, L. M., and Barnhart, J. L., *J. Dairy Sci.*, **15**, 401 (1932).
399. Timasheff, S. N., and Townend, R., *J. Am. Chem. Soc.*, **80**, 4433 (1958).
400. Tobias, J., Whitney, R. McL., and Tracy, P. H., *J. Dairy Sci.*, **35**, 1036 (1952).
401. Torboli, A., *Boll. Soc. Ital. Biol. Sper.*, **20**, 445 (1945).
402. Townley, V. H., M. S. Thesis, Univ. of Minn. (**1950**), Ph.D. Thesis, Univ. of Minn., Minneapolis (**1953**).
403. Townend, R., and Timasheff, S. N., Paper #146, *131st Mtg. Am. Chem. Soc.*, **1957**.
404. Towson, A. M., and Trout, G. M., *Food Res.*, **11**, 261 (1946).
405. Tracy, P. H., and Ruehe, H. A., *Ill. Agr. Expt. Sta. Bull.*, **352**, 569 (1930).
406. Trautman, J. C., Ph.D. Thesis, Univ. of Wisc., Madison (**1958**).
407. Trout, G. M., "Homogenized Milk," p. 30, Michigan State College Press, East Lansing (**1950**).
408. Tumerman, L., Fram, H., and Cornely, K. W., *J. Dairy Sci.*, **37**, 830 (1954).
409. Vakaleris, D. G., U.S. patent 3,039,879, June 19, 1962.
410. Van Kreveld, A., *12th Intern. Dairy Congr. (Stockholm), Proc.*, **2**, 44 (1949) also: *Neth. Milk Dairy J.*, **4**, 115 (1950).
411. Van Kreveld, A., and Van Minnen, G., Abst. paper *Am. Chem. Soc.*, Sept., 1955, p. 7A.
412. Van Kreveld, A., and Minnen, G., Van, *Neth. Milk and Dairy J.*, **9**, 1 (1955).
413. Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, **14**, 211 (1913).
414. Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, **20**, 135 (1915).
415. Van Slyke, L. L., and Keeler, R. F., *N.Y. (Geneva) Agr. Exp. Sta., Tech. Bull.*, **78** (1920).
416. Vas, K., *Wien., Chem.-Ztg.*, **47**, 49 (1944).
417. Verma, I. S., and Gehrke, C. W., *J. Dairy Sci.*, **40**, 1366 (1957).
418. Verma, I. S., and Gehrke, C. W., *15th Intern. Dairy Congr., London, Proc.*, **2**, 648 (1959).
419. Verma, I. S., and Sommer, H. H., *J. Dairy Sci.*, **33**, 397 (1950).
420. Vlodavets, I. N., and Shtal'berg, S., *Molochnaya Prom.*, **15**, No. 3, 35 (1954).
421. von Hippel, P. H., and Waugh, D. F., *J. Am. Chem. Soc.*, **77**, 4311 (1955).
422. von Hippel, P. H., and Waugh, D. F., *Fed. Proc.*, **15**, 194 (1956).
423. Waite, R., and White, J. C. D., *J. Dairy Res.*, **16**, 379 (1949).
424. Warner, R. C., *J. Am. Chem. Soc.*, **66**, 1725 (1944).
425. Wake, R. G., *Australian J. Sci.*, **20**, 147 (1957).
426. Wake, R. G., *Australian J. Biol. Sci.*, **12**, 479 (1959).
427. Wake, R. G., and Baldwin, R. L., *Biochim. et Biophys. Acta*, **47**, 225 (1961).
428. Wallgren, K. E. L., Ph.D. Thesis, Department Dairy and Food Industry, University of Wisconsin, Madison (**1961**).
429. Wannow, H. A., *Kolloid-Beihefte*, **50**, 367 (1939).
430. Wannow, H. A., *Kolloid Z.*, **87**, 311 (1939).
431. Warner, R. C., *J. Am. Chem. Soc.*, **66**, 1725 (1944).
432. Waugh, D. F., "*Adv. Protein Chem.*," **9**, 325 (1954) Academic Press, N. Y.

433. Waugh, D. F., *J. Phys. Chem.*, **65**, 1793 (1961).
434. Waugh, D. F., *Dis. Faraday Society*, **25**, 186 (1958).
435. Waugh, D. F., and von Hippel, P. H., *J. Am. Chem. Soc.*, **78**, 4576 (1956).
436. Webb, B. H., *J. Dairy Sci.*, **18**, 81 (1935).
437. Webb B. H., *J. Dairy Sci.*, **14**, 508 (1931).
438. Webb, B. H., *J. Dairy Sci.*, **11**, 471 (1928).
439. Webb, B. H., and Bell, R. W., *J. Dairy Sci.*, **25**, 301 (1942).
440. Webb, B. H., and Bell, R. W., *J. Dairy Sci.*, **26**, 1071 (1943).
441. Webb, B. H., Bell, R. W., Deysher, E. F., and Holm, G. E., *J. Dairy Sci.*, **26**, 571 (1943).
442. Webb, B. H., Deysher, E. F., and Potter, F. E., *J. Dairy Sci.*, **34**, 1111 (1951).
443. Webb, B. H., and Holm, G. E., *J. Dairy Sci.*, **11**, 243 (1928).
444. Webb, B. H., and Holm, G. E., *J. Dairy Sci.*, **13**, 25 (1930).
445. Webb, B. H., and Holm, G. E., *J. Dairy Sci.*, **15**, 345 (1932).
446. Webb, B. H., and Hufnagel, C. F., *J. Dairy Sci.*, **29**, 221 (1946).
447. Webb, B. H., and Hufnagel, C. F., U.S. Dept. Agr., Bur. Dairy Ind., *BDIM-Inf*-**47** (1947).
448. Webb, B. H., and Hufnagel, C. F., U.S. Dept. Agr., Bur. Dairy Ind. *BDIM-Inf*-**61** (June 14, 1938).
449. Webb, B. H., and Hufnagel, C. F., *J. Dairy Sci.*, **31**, 21 (1948).
450. Whitaker, R., Sherman, J. M., and Sharp, P. F., *J. Dairy Sci.*, **10**, 361 (1927).
451. White, J. C. D., and Davies, D. T., *J. Dairy Res.*, **25**, 256 (1958).
452. White, J. C. D., and Davies, D. T., *J. Dairy Res.*, **25**, 267 (1958).
453. White, J. C. D., and Davies, D. T., *J. Dairy Res.*, **25**, 281 (1958).
454. Whitney, R. McL., Paulson, K., and Murthy, G. K., *J. Dairy Sci.*, **35**, 937 (1952).
455. Whittier, E. O., and Benton, A. G., *J. Dairy Sci.*, **9**, 481 (1926).
456. Whittier, E. O., and Benton, A. G., *J. Dairy Sci.*, **10**, 126 (1927).
457. Whittier, E. O., and Webb, B. H., "By-Products from Milk." Reinhold Publishing Corp., New York, p. 191 (**1950**).
458. Wiegner, G., *Z. Nahr. Genussm.*, **27**, 425 (1914).
459. Wilcox, D. F., U.S. patent 2,845,350, July 29, 1958.
460. Wilcox, D. F., U.S. patent 2,860,057, Nov. 11, 1958.
461. Wilson, J. A., *J. Am. Chem. Soc.*, **38**, 1982 (1916).
462. Wilson, H. K., and Herreid, E. O., *J. Dairy Sci.*, **41**, 702 (1958).
463. Wilson, H. K., and Herreid, E. O., *J. Dairy Sci.*, **41**, 1287 (1958).
464. Wilson, H. K., and Herreid, E. O., *J. Dairy Sci.*, **43**, 850 (1960).
465. Wilson, H. K., and Herreid, E. O., *J. Dairy Sci.*, **44**, 552 (1961).
466. Wilson, H. K., Herreid, E. O., and Whitney, R. McL., *J. Dairy Sci.*, **43**, 165 (1960).
467. Wilson, H. K., Yoshino, U., and Herreid, E. O., *J. Dairy Sci.*, **44**, 1155 (1961).
468. Wohlisch, E., and Juhling, L., *Biochem. Z.*, **297**, 353 (1938).
469. Wright, N. C., *Biochem. J.*, **18**, 245 (1924).
470. Wright, N. C., *J. Dairy Res.*, **4**, 122 (1932).
471. Wright, R. C., and Tramer, J., *J. Dairy Res.*, **20**, 177 (1953).
472. Wright, R. C., and Tramer, J., *J. Dairy Res.*, **20**, 258 (1953).
473. Wright, R. C., and Tramer, J., *J. Dairy Res.*, **23**, 248 (1956).
474. Wu, H., *Chinese J. Physiol.*, **5**, 321 (1931).
475. Zhdanova, E. A., and Vlodavets, I. N., *Biokhimiia*, **24**, 398 (1959).
476. Zittle, C. A., and Bingham, E. W., *J. Dairy Sci.*, **42**, 1772 (1959).
477. Zittle, C. A., and DellaMonica, E. S., *J. Dairy Sci.*, **39**, 514 (1956).
478. Zittle, C. A., DellaMonica, E. S., and Custer, J. H., *J. Dairy Sci.*, **39**, 1651 (1956).
479. Zittle, C. A., DellaMonica, E. S., and Custer, J. H., *J. Dairy Sci.*, **40**, 280 (1957).
480. Zittle, C. A., DellaMonica, E. S., Rudd, R. K., and Custer, J. H., *J. Am. Chem. Soc.*, **79**, 4661 (1957).
481. Zittle, C. A., DellaMonica, E. S., Rudd, R. K., and Custer, J. H., *Arch. Biochem. and Biophys.*, **76**, 342 (1958).
482. Zittle, C. A., and Pepper, L., *J. Dairy Sci.*, **41**, 1671 (1958).
483. Zweig, G., and Block, R. J., *J. Dairy Sci.*, **36**, 427 (1953).